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Molecular biology and functions of epilysin (MMP-28)

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Academic Dissertation

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Original publications

This thesis is based on the following original articles, which are referred to by their Roman numerals in the text.

I. Illman, S.A., Keski-Oja, J., and Lohi, J.: Promoter characterization of the human and mouse epilysin (MMP-28) genes. **Gene 275,** 185-194, 2001.

II. Illman, S.A., Keski-Oja, J., Parks, W.C., and Lohi, J.: The mouse matrix metalloproteinase, epilysin (MMP-28), is alternatively spliced and processed by a furin-like proprotein convertase. **Biochem. J. 375,** 191-197, 2003.

III. Illman, S.A., Lehti, K., Keski-Oja, J., and Lohi, J.: Epilysin (MMP-28) induces TGF-β mediated epithelial to mesenchymal transition in lung carcinoma cells. **J. Cell Sci. 119,** 3856-3865, 2006.

Abbreviations

aa	amino acid
ADAM	a disintegrin-like and metalloprotease domain
ADAMTS	ADAM with trombospondin type 1 motifs
AP	activating protein
ATP	adenosine triphosphate
bHLH	basic helix-loop-helix
BM	basement membrane
bp	base pair
CNS	central nervous system
cpm	counts per minute
D-MEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
E	embryonic day
ECM	extracellular matrix
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
EMMPRIN	extracellular matrix metalloproteinase inducer
EMT	epithelial to mesenchymal transition
ETS	E26 transformation-specific
FCS	fetal calf serum
FGF	fibroblast growth factor
GAG	glycosaminoglycan
GAPDH	glyceraldehyde-3-phosphatase dehydrogenase
GMSA	gel mobility shift assay
GPI	glycosyl phosphatidyl inositol
HAT	histone acetyltransferase
HDAC	histone deacetylase
HE	hematoxylin and eosin
HGF	hepatocyte growth factor
ICAM	intracellular adhesion molecule
IGF	insulin-like growth factor
IGFBP	IGF binding protein
IgG	immunoglobulin G
IL	interleukin
kb	kilobase
KC	keratinocyte-derived chemokine
kDa	kilodalton
KGF	keratinocyte growth factor
KO	knock-out
LAP	latency associated peptide
LTBP	latent TGF-β binding protein
MAPK	mitogen-activated protein kinase
MDCK	Madin-Darby canine kidney
MEM	Eagle's minimal essential medium
MMP	matrix metalloproteinase
MT-MMP	membrane type MMP
mRNA	messenger RNA
NCAM	neural cell adhesion molecule

NF-κB	nuclear factor κB
NGF	nerve growth factor
nt	nucleotide
OA	osteoarthritis
OSCC	oral squamous cell carcinoma
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PEA	phosphoprotein enriched in astrocytes
PFA	paraformaldehyde
PG	proteoglycan
PI3K	phosphatidylinositol 3 kinase
RECK	reversion-inducing cysteine-rich protein with Kazal motifs
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
SCC	squamous cell carcinoma
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sip	Smad-interacting protein
siRNA	small interfering RNA
SNP	single-nucleotide polymorphism
Sox	sex-determining region Y-type HMG box proteins
Sp	specificity protein
Sry	sex-determining region Y gene
STRAP	serine threonine receptor associated protein
TACE	TNF-α converting enzyme
TGF-β	transforming growth factor β
TIMP	tissue inhibitor of metalloproteinase
TNF	tumor necrosis factor
uPA	urokinase-type plasminogen activator
uPAR	uPA receptor
UTR	untranslated region
VEGF	vascular endothelial growth factor
wt	wild type
XMMP	*Xenopus laevis* MMP

Abstract

Epilysin (MMP-28) is the most recently identified member of the matrix metalloproteinase (MMP) family of extracellular proteases. Together these enzymes are capable of degrading almost all components of the extracellular matrix (ECM) and are thus involved in important biological processes such as development, wound healing and immune functions, but also in pathological processes such as tumor invasion, metastasis and arthritis. MMPs do not act solely by degrading the ECM. They also regulate cell behavior by releasing growth factors and biologically active peptides from the ECM, by modulating cell surface receptors and adhesion molecules and by regulating the activity of many important mediators in inflammatory pathways. The aim of this study was to define the unique role of epilysin within the MMP-family, to elucidate how and when it is expressed and how its catalytic activity is regulated. To gain information on its essential functions and substrates, the specific aim was to characterize how epilysin affects the phenotype of epithelial cells, where it is biologically expressed.

Applying different promoter analysis techniques we found that the *epilysin* promoter contains a well conserved GT-box that is essential for the basic expression of this gene. Transcription factors Sp1 and Sp3 bind this sequence and could hence regulate both the basic and cell type and differentiation stage specific expression of epilysin. We cloned mouse epilysin cDNA and found that epilysin is well conserved between human and mouse genomes and that epilysin is glycosylated and activated by furin. RNA-analysis revealed that epilysin, similarly to in human tissues, is normally expressed in a number of mouse tissues. The expression pattern differs from most other MMPs, which are expressed only in response to injury or inflammation and in pathological processes like cancer. These findings implicate that epilysin could be involved in tissue homeostasis, perhaps fine-tuning the phenotype of epithelial cells according to signals from the ECM.

In view of these results, it was unexpected to find that epilysin can induce a stable epithelial to mesenchymal transition (EMT) when overexpressed in epithelial lung carcinoma cells. Transforming growth factor β (TGF-β) was recognized as a crucial mediator of this process, which was characterized by the loss of E-cadherin mediated cell-cell adhesion, elevated expression of gelatinase B and MT1-MMP and increased cell migration and invasion into collagen I gels. We also observed that epilysin is bound to the surface of epithelial cells and that this interaction is lost upon cell transformation and is susceptible to degradation by membrane type-1-MMP (MT1-MMP). The wide expression of epilysin under physiological conditions implicates that its effects on epithelial cell phenotype *in vivo* are not as dramatic as seen in our *in vitro* cell system. Nevertheless, current results indicate a possible interaction between epilysin and TGF-β also under physiological circumstances, where epilysin activity may not induce EMT but, instead, trigger less permanent changes in TGF-β signaling and cell motility. Epilysin may thus play an important role in TGF-β regulated events such as wound healing and inflammation, processes where involvement of epilysin has been indicated.

1 Introduction

1.1 Extracellular matrix

All tissues are formed of individual cells that are held together by the surrounding ECM (Aumailley and Gayraud, 1998; Zagris, 2001). The molecules of the ECM not only confer mechanical support as they also interact with the cells to regulate cell adhesion and guide cell migration. In addition, the ECM functions as storage for signaling molecules, growth factors and enzymes modulating their activity and availability. The ECM thus provides a dynamic environment for the cells embedded within and plays a key role in determining cell phenotype (Streuli, 1999).

The ECM is a complex structure consisting of numerous components. As the ECM proteins are produced by the cells it harbors such as fibroblasts, osteoblasts, and macrophages, the composition and architecture of the ECM varies largely between different tissues (Aumailley and Gayraud, 1998). ECM can structurally be divided into two major components, the interstitial matrix and the basement membrane (BM).

1.1.1 Interstitial matrix

The interstitial matrix is formed by a network of fibers surrounded by a gel-like substance containing glycosaminoglycans (GAGs), proteoglycans (PGs) and water that these molecules hold. The most abundant structural components of the interstitial matrix are the collagens, which are trimeric molecules containing three identical or different α-chains. The 43 different α-chains can be combined in numerous different ways to form a wide variety of different fibrillar and non-fibrillar collagen types, of which 28 different types have been identified to date (Myllyharju and Kivirikko, 2004; Ricard-Blum and Ruggiero, 2005; Kadler *et al.*, 2007). The fibrillar collagens (types I, II, III, V, XI, XXIV and XXVI) typically consist of three α-chains containing a variable number of Gly-X-Y repeats that form a tight triple-helix. The fibrillar collagens confer mechanical strength to tissues by assembling into large cross-linked fibrils. The α-chains are synthesized and secreted as precursors with large N- and C-terminal propeptides that prevent them from folding prematurely. The triple-helical structure is highly resistant to proteolysis and degradation. Type I collagen is the most abundant collagen in most connective tissues. It consists of two α1(I) and one α2(I) chain and is designated $[\alpha1(I)]_2\alpha2(I)$. The tissue distribution of other fibrillar collagens is much more restricted.

The non-fibrillar collagens constitute a more heterogeneous group of molecules, and are further divided into several subgroups based on their function, supramolecular assemblies or other features (Myllyharju and Kivirikko, 2004; Ricard-Blum and Ruggiero, 2005; Kadler *et al.*, 2007). Of the network-forming collagens (types IV, VIII and X), type IV is the major component of BMs (see paragraph 1.1.2). The fibril-associated and related collagens (types IX, XII, XIV, XVI, XIX, XX, XXI and XXII) do not form fibers by themselves but are found attached to the surface of fibrils of the fibrillar collagens. In addition there are beaded-filament-forming collagens (types VI, XXVI and XXVIII), which form microfibrils, and one anchoring collagen (type VII), which participates in the attachment of BM and epithelia to underlying stroma. Collagens VI and VII are found in most connective tissues, whereas the expression of collagens XXVI and XXVIII is more restricted. Further, there are collagens with transmembrane domains (types XIII, XVII, XXIII and XXV), which participate in cell adhesion. The closely related multiplexins (multiple triple-helix domain and interruptions,

types XV and XVIII) are found in attachment to the BM. Proteolytic cleavage of the C-terminal domain of collagen XVIII generates the antiangiogenic peptide endostatin (O´Reilly *et al.*, 1997; Wickström *et al.*, 2005).

Some tissues like the lung and blood vessels, contain elastic fibers (Arribas *et al.*, 2006), which allow these tissues to endure stretch and pressure. The elastic fibers are composed mainly of elastin that is first produced as a soluble precursor, tropoelastin, which is then converted to highly insoluble elastin upon secretion from the cells (Mecham and Heuser, 1991). Elastin is extensively cross-linked to form stable fibers. These fibers are surrounded by microfibrils constructed of fibrillin and other associated proteins, such as the latent TGF-β binding proteins (LTBPs) (Hyytiäinen *et al.*, 2004).

Various proteoglycans (PGs) are also important components of the interstitial ECM They are highly hydrophilic and retain water and thus formi the amorphous ground substance of the ECM (Iozzo, 1998). The PGs consist of a core protein attaching numerous sulfated sugar chains, glycosaminoglycans (GAGs), which constitute the major part of the overall weight of the PGs. In addition to retaining water, the PGs possess more specialized features. They influence cell growth and maturation of specialized tissues, modulate growth factor availability and binding of these to cognate receptors, and affect tumor cell growth and invasion (Gesslbauer *et al.*, 2007). For example, the heparan sulfate PGs bind several ECM associated growth factors of the fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) families. Different cell types express different PGs, whose GAGs may vary in response to growth factors during developmental and pathological processes. The proteoglycanome, which is the entire subset of proteoglycans expressed in a tissue, thus plays an active role in modulating cell phenotype and tissue composition and function.

Glycoproteins, another important group of ECM molecules, differ from the PGs in that their carbohydrate component constitutes a much smaller proportion of the overall molecule. One of the most abundant glycoproteins is fibronectin (Ruoslahti and Vaheri, 1974), which is a large glycoprotein containing two disulfide linked subunits of 250-280 kDa each. Fibronectin is located in fibrillar structures, especially in provisional matrices during wound healing, but is also widely distributed in soluble form in the plasma. Fibronectin interacts with numerous proteins on the cell surface as well as in the ECM (Magnusson and Mosher, 1998).

1.1.2 Basement membrane

BMs form highly specialized sheets of ECM proteins that separate epithelial and endothelial cells from the underlying stromal tissue. In addition to serving as an adhesion interface between these different compartments, the BM also functions as a permeability barrier and controls cell organization and differentiation through interactions with cell surface receptors and ECM proteins (Masunaga, 2006). The major structural component of the BM is collagen IV, which is a heterotrimer that forms flexible helical rods with interruptions. The most common structure of collagen IV is $[\alpha1(IV)]_2\alpha2(IV)$, but six different α-chains have been identified that can be combined into a number of different trimers (Hudson *et al.*, 2003). The trimers self-assemble through different forms of intra and intermolecular interactions into a highly cross linked network that confers mechanical stability to the BM (Timpl, 1996).

The most abundant glycoproteins of the BM are laminins, which are formed through the association of three genetically different polypeptides, the α, β and γ chains. Five α, four β and three γ peptides have been identified, which can associate to form at least 15 different heterotrimers that form cross-shaped molecules with three short arms (Patarroyo *et al.*, 2002; Miner and Yurchenco, 2004). In the presence of calcium, laminin I self-assembles into a polymeric scaffold that is further connected to the collagen IV network via the glycoprotein

nidogen. Nidogen further interacts with other glycoproteins like fibulin and the abundant BM proteoglycan perlecan (Aumailley and Gayraud, 1998).

1.1.3 Interactions between cells and the ECM

The components of the ECM are in constant interaction with the surrounding cells. ECM can affect cell behavior either through the growth factors that it harbors or through direct interactions between ECM molecules and cell surface receptors (Nelson and Bissell, 2006). The major cell surface receptors mediating cell adhesion to ECM proteins are the integrins (Hynes, 2002). In addition to binding ECM proteins in the extracellular compartment, integrins also form transmembrane contacts with intracellular proteins and the cytoskeleton. The integrins consequently transmit signals in two directions to coordinate the intracellular environment with extracellular signals, and in this way play critical roles in numerous biological as well as pathological processes. The integrins are heterodimers formed by noncovalent association of an α and a β subunit. In mammals 18 α subunits and 8 β subunits have been identified that can be combined to form over 20 different integrins with specific tissue distribution and ligand preferences (Heino, 2000; Hynes, 2002).

During embryonic development the interactions between embryonic cells and their ECM initiate and guide developmental processes, such as cell migration, morphogenesis, and modulation of growth and differentiation pathways (Zagris, 2001). In adult tissues the interplay between cells and the ECM is more stable emphasizing cell adhesion and restraining cell migration, which is important for tissue homeostasis and organ function (Geiger *et al.*, 2001). An important ECM molecule promoting cell adhesion is fibronectin (Ruoslahti and Vaheri, 1974; Magnusson and Mosher, 1998). Its adhesive activity has been traced to an RGD (Arg-Gly-Asp) sequence commonly found also in other ECM molecules functioning in cell adhesion, such as tenascin and laminin I (Aumailley and Gayraud, 1998).

During tissue level processes such as inflammation, wound healing and metastasis, novel signals are generated from the ECM and new epitopes exposed within the surrounding tissues. These induce the cells to react properly to the new circumstances (Gustafsson and Fässler, 2000). The ability of any cell to remodel the ECM is therefore critical for interactions with its surroundings and, on a larger scale, for any organism to function properly (Vu and Werb, 2000; Sternlicht and Werb, 2001; Stamenkovic, 2003). Numerous proteases have been implicated in the remodeling and degradation of the ECM. These can be categorized either as exopeptidases that cleave their substrates at terminal sites or as endopeptidases that cleave at internal sites. Based on their catalytic mechanism endopeptidases can be divided into four major classes: serine, cysteine, aspartic and metalloproteinases. Most of the metalloproteinases take advantage of zinc for their catalytic activity. This large family is further divided into subfamilies, one of which is the metzincins, which contains the family of matrix metalloproteinases (MMPs) (Woessner, 1998).

1.2 Matrix Metalloproteinases

The first evidence of a soluble enzyme capable of degrading native collagen came from experiments, where tissue fragments of involuting tadpole tails were cultured on native collagen gels (Gross and Lapiere, 1962). Since then a family of 24 human endopeptidases capable of degrading most ECM components, as well as several cell surface and pericellular proteins, has been identified (Sternlicht and Werb, 2001). MMPs not only possess the means to degrade ECM, but also to release bioactive fragments and growth factors, thus influencing fundamental biological and pathological processes like embryonic development, tissue

morphogenesis, wound repair, inflammation and cancer (Egeblad and Werb, 2002; Mott and Werb, 2004). All MMPs have distinct but partially overlapping functions (Sternlicht and Werb, 2001). The reason for this redundancy may be to protect the organism from the loss of regulatory control, as MMPs participate in such a wide variety of essential processes. This has become evident through the generation of genetically modified mice lacking specific *Mmp* genes. Many of these animals exhibit an unexpectedly mild phenotype, compared to what could be expected from the knowledge of their *in vitro* substrates (Table II).

1.2.1 Structures and substrates of the different MMP subfamilies

MMPs share a common domain structure and catalytic mechanism (Nagase *et al.*, 2006). All MMPs are extracellular proteins containing an N-terminal signal sequence that targets them for secretion. The signal sequence is followed by a propeptide containing a conserved PRCGXPD sequence and the catalytic domain that contains a conserved HEXXHXXGXXH sequence. This sequence includs three histidine residues that together bind a zinc ion critical for the catalytic activity. Before removal of the propeptide its conserved cysteine residue also interacts with this zinc ion, preventing catalytic activity. This mechanism has been named the "cysteine switch" (Van Wart and Birkedal-Hansen, 1990). Specific amino acids within the catalytic domain define substrate specificity by allowing interactions with a limited subset of substrates only (Visse and Nagase, 2003). Substrate specificity is further determined by substrate binding sites located outside the catalytic domain named exosites. The catalytic domain in most MMPs is followed by a proline-rich linker, the hinge region. This is further attached to the C-terminal hemopexin domain, which functions in substrate and inhibitor interactions. Exceptions are the matrilysins MMP-7 (Muller *et al.*, 1988) and MMP-26 (Park *et al.*, 2000) that lack the hemopexin domain, and MMP-23 which contains C-terminal cysteine- and proline-rich domains and a domain resembling the interleukin (IL)-1 type II receptor instead of the hemopexin domain (Pei, 1999a; Velasco *et al.*, 1999). In addition to the common domains, MMPs contain a number of more specialized structures. Based on these specific characteristics and substrate preference, MMPs can be further divided into subfamilies (Fig. 1, Table I).

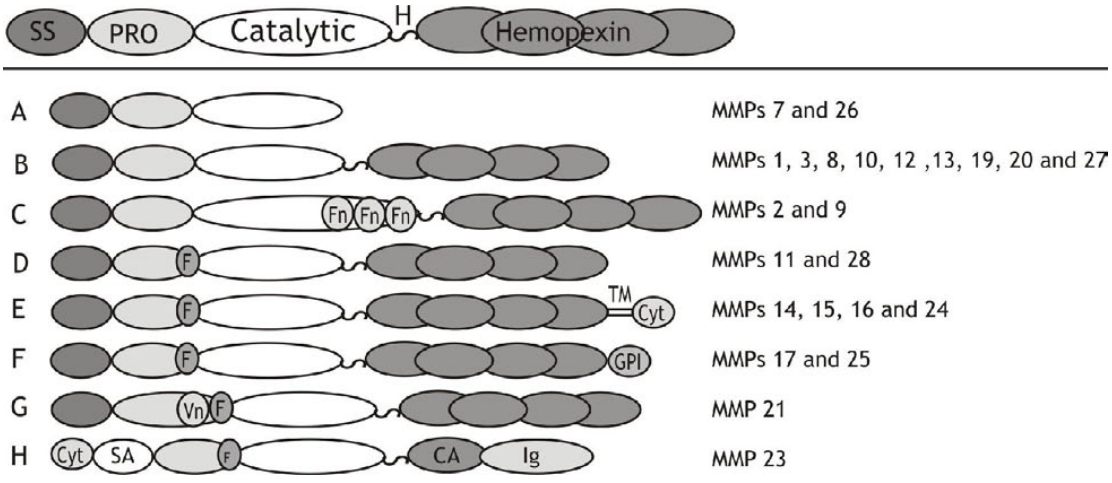


Figure 1. **Domain structures of the MMP subfamilies**. Abbreviations: SS, signal sequence; PRO, propeptide; Catalytic, catalytic domain; H, hinge region; Hemopexin, hemopexin domain; Fn, fibronectin type II inserts; F, furin-cleavage site; TM, transmembrane domain; Cyt, cytoplasmic domain; GPI, glycosylphosphatidyl inositol-anchoring domain; Vn, vitronectin-like sequence; CA, cysteine array; Ig, immunoglobulin-like domain. Modified from Sternlicht and Werb, 2001; Ala-aho and Kähäri, 2005.

Table I. Substrates of human MMPs. MMPs are listed together with their common names. DS: domain structure (refers to DS outlined in Fig. 1). Some substrates are listed in the right column.

	Name	DS	Substrates
MMP-1	collagenase-1	B	col I-III VII VIII X XI, aggrecan, gelatin, fibronectin, laminin, nidogen, IGFBPs, tenascin, vitronectin, fibrin, fibrinogen, casein, pro-TNFα, IL-1β, α1-PI, α2-M, pro-MMP-1 and -2.
MMP-2	gelatinase A	C	col I III-V VII X XI, aggrecan, elastin, fibrillin, fibronectin, gelatin, nidogen, IGFBPs, laminin, tenascin, vitronectin, α1-PI, α2-M, fibrin, fibrinogen, IL-1β, pro-TGFβ, pro-TNF-α, plasminogen, pro-MMP-9, and -13, dystroglycan.
MMP-3	stromelysin-1	B	col III-V VII X XI, elastin, aggrecan, nidogen, fibrillin, fibronectin, gelatin, IGFBPs, laminin, tenascin, vitronectin, α1-PI, α2-M, E-cadherin, fibrin, fibrinogen, casein, IL-1β, pro-TNF-α, plasminogen, uPA, pro-MMP-1, -2, -8, -9, and -13.
MMP-7	matrilysin	A	col I IV, aggrecan, elastin, nidogen, gelatin, fibronectin, laminin, tenascin, vitronectin, E-cadherin, pro-α-defensin, fibrinogen, casein, pro-TNF-α, plasminogen, α1-PI, Fas-ligand, syndecan-1, pro-MMP-1, -2, and -9.
MMP-8	collagenase-2	B	col I-III, aggrecan, α1-PI, α2-M, fibrinogen, pro-MMP-8.
MMP-9	gelatinase B	C	col IV V XI XIV, aggrecan, elastin, fibrillin, gelatin, laminin, vitronectin, α1-PI, α2-M, fibrin, fibrinogen, casein, IL-1β, pro-TGFβ, pro-TNF-α, plasminogen, E-cadherin, dystroglycan.
MMP-10	stromelysin-2	B	col III-V, aggrecan, elastin, fibronectin, gelatin, fibrinogen, casein, laminin-5, pro-MMP-1, -2, -8, and -13.
MMP-11	stromelysin-3	D	IGFBPs, α1-PI, α2-M.
MMP-12	metalloelastase	B	col I IV, aggrecan, elastin, nidogen, fibrillin, fibronectin, gelatin, α1-PI, α2-M, fibrinogen, pro-TNF-α, plasminogen, pro-MMP-2.
MMP-13	collagenase-3	B	col I-III VI IX X XIV, aggrecan, fibrillin, fibronectin, gelatin, α2-M, fibrinogen, casein, pro-MMP-2, -9, and -13.
MMP-14	MT1-MMP	E	col I-III, aggrecan, fibrillin, fibronectin, nidogen, gelatin, laminin, vitronectin, α1-PI, α-2M, fibrin, fibrinogen, pro-TNF-α, CD44, α_V integrin, syndecan-1, EMMPRIN, E- and N-cadherins, ICAM-1, pro-MMP-2, and -13.
MMP-15	MT2-MMP	E	col I, fibrinogen, pro-MMP-2, and -13.
MMP-16	MT3-MMP	E	col III, fibronectin, pro-MMP-2.
MMP-17	MT4-MMP	F	gelatin, fibrinogen, fibrin, pro-TNF-α, ADAMTS4.
MMP-19	RASI	B	col I IV, fibronectin, gelatin, IGFBPs, tenascin, casein.
MMP-20	enamelysin	B	amelogenin.
MMP-21	XMMP	G	α1-PI.
MMP-23		H	
MMP-24	MT5-MMP	E	gelatin, pro-MMP-2.
MMP-25	MT6-MMP	F	col IV, gelatin, fibrin, fibronectin, chondroitin sulfate proteoglycan, dermatan sulfate proteoglycan, α1-PI.
MMP-26	matrilysin-2	A	col IV, fibronectin, gelatin, fibrinogen, α1-PI, pro-MMP-9.
MMP-27		B	casein, gelatin.
MMP-28	epilysin	D	casein, NCAM.

Col denotes collagen; α1-PI, α1-proteinase inhibitor; α2-M, α2-macroglobulin; IL-1β, interleukin-1β; uPA, urokinase-type plasminogen activator. Based on Vu and Werb, 2000; English *et al.*, 2001; Sternlicht and Werb 2001; Ala-aho and Kähäri, 2004; Gao *et al.*, 2004; Krampert *et al.*, 2004; Wielockx *et al.*, 2004; Agrawal *et al.*, 2006; Covington *et al.* 2006; Egawa *et al.*, 2006; Itoh and Seiki, 2006; Sithu *et al.*,2007; Symowicz *et al.*, 2007; Werner *et al.*, 2007.

Accordingly, MMPs also share a similar gene arrangement suggesting that they have evolved from a common ancestor gene. At least eight of the known human *Mmp* genes are clustered on chromosome 11 at 11q21-23.

1.2.1.1 Collagenases

Collagenases 1-4 cleave interstitial collagens I, II and III into two fragments comprising 3/4 and 1/4 of the molecule, respectively (Jeffrey, 1998). The different collagenases, however, have different substrate preferences as collagenase-1 preferentially cleaves collagen III whereas collagenase-2 prefers collagen I. The collagenases also digest a number of other ECM components and other molecules (Table I, Sternlicht and Werb, 2001).

Collagenase-1 (MMP-1) is the founding member of the MMP-family (Gross and Lapiere, 1962). The protein and the cDNA were originally identified and purified from human skin fibroblasts (Stricklin *et al.*, 1977; Goldberg *et al.*, 1986), but *in vitro* the enzyme is also secreted by other fibroblasts, chondrocytes, osteoblasts, endothelial cells and tumor cells, among others (Ala-aho and Kähäri, 2005). Collagenase-2 (MMP-8) has also been named neutrophil collagenase as it is synthesized by neutrophils during their maturation in bone marrow and stored in specific granules within the cells, from where the enzyme can be released upon external signals (Hasty *et al.*, 1990). Collagenase-2 is also expressed in human articular cartilage, in rheumatoid synovium and during bronchitis (Ala-aho and Kähäri, 2005). Collagenase-3 (MMP-13) preferentially cleaves collagen II found in cartilage but has also strong gelatinolytic activity. It has been implicated in biological processes like organ development as well as in pathological processes related to chronic wounds and cancer (Ala-aho and Kähäri, 2005). Collagenase IV (MMP-18) is expressed only in *Xenopus laevis* (Stolow *et al.*, 1996).

The individual roles of the collagenases in rodents differ from those in humans. The primary interstitial collagenases of these animals show highest homology to human collagenase-3 instead of the major human collagenase, collagenase-1. The expression profiles of mouse and rat collagenase-3 are, however, highly similar to that of human collagenase-1, indicating that these MMPs are functional homologues. Two closely related mouse counterparts to human collagenase-1, Mcol-A and Mcol-B, have been cloned (Balbín *et al.*, 2001). Their expression is, however, restricted to embryo implantation and only Mcol-A is able to cleave collagen I.

1.2.1.2 Gelatinases

The gelatinases act on a large number of substrates including collagens, elastin, fibronectin and denatured collagen, gelatin. In addition, they cleave numerous non-ECM molecules *in vitro* (Table I, Sternlicht and Werb, 2001). The enzymes are distinguished by three head-to-tail repeats of a type II fibronectin domain in their catalytic domains (Fig. 1), which are required for the gelatinases to bind and digest collagens and elastin (Murphy *et al.*, 1994; Shipley *et al.*, 1996). Gelatinase A (MMP-2) differs from most other MMPs in the sense that it is expressed constitutively by numerous normal and transformed cells and has ubiquitous tissue distribution (Yu *et al.*, 1998). The expression of gelatinase B (MMP-9) is more restricted. It is produced by neutrophils and stored within secretory granules, from where the enzyme can be released upon demand. Gelatinase B is also secreted by macrophages and various tumors and transformed cell lines and mostly maintained at low levels in normal tissues (Vu and Werb, 1998). Both gelatinases are, however, frequently upregulated in different forms of cancers, especially during tumor metastasis (Egeblad and Werb, 2002; Deryugina and Quigley, 2006).

1.2.1.3 Stromelysins

The domain structure of the stromelysins resembles that of the collagenases (Fig. 1), but they cannot cleave interstitial collagens. Stromelysins 1 (MMP-3) and 2 (MMP-10) are similar in structure and substrate specificity, whereas stromelysin-3 (MMP-11) differs from these two in both aspects. Stromelysins 1 and 2 degrade numerous ECM proteins and participate in the activation of other MMPs (Table I, Sternlicht *et al.*, 1999). Stromelysin-3 shows only weak activity towards most ECM proteins *in vitro* but, instead, cleaves and inactivates proteinase inhibitors (Pei *et al.*, 1994) and insulin-like growth factor binding protein-1 (IGFBP-1) (Manes *et al.*, 1997). Stromelysin-3 contains an RXKR sequence at the end of the prodomain, which serves as a recognition site for the proprotein convertase furin, enabling intracellular activation of the enzyme (Pei and Weiss, 1995).

1.2.1.4 Matrilysins

Matrilysins are the smallest MMPs since they lack the hemopexin domain. Matrilysin (MMP-7) is synthesized by epithelial cells and is secreted apically. Matrilysin is, unlike most other MMPs, expressed in normal adult tissues (Wielockx *et al.*, 2004) and it cleaves a large variety of both collagens and other ECM proteins *in vitro* (Table I). Matrilysin activates intestinal crypt α-defensins, which are anti-microbial peptides that participate in the innate immune system of the intestine (Table II, Wilson *et al.*, 1999). Matrilysin also cleaves important cell surface adhesions receptors like E-cadherin (Noë *et al.*, 2001), implicating its role in cell migration (McGuire *et al.*, 2003). Matrilysin is further frequently expressed in different forms and stages of cancer and in inflammatory disorders (Wielockx *et al.*, 2004).

Endometase (matrilysin-2, MMP-26), which digests several ECM proteins, is expressed in normal endometrium and in some tumors (Park *et al.*, 2000; Uria and Lopez-Otin, 2000). Endometase differs form all other MMPs in the respect that it contains an estrogen-response element in the promoter and can be induced in hormone regulated carcinomas (Li *et al.*, 2004b).

1.2.1.5 Membrane-type MMPs

Four of the MMPs, namely MT1-, MT2-, MT3-, and MT5-MMP (MMPs 14, 15, 16 and 24) have a C-terminal transmembrane domain and a short intracellular tail, which target them to the cell membrane (Fig. 1). MT4- and MT6-MMP (MMPs 17 and 25) are attached to the cell surface by C-terminal glycosylphosphatidylinositol (GPI) anchors (Fig. 1). All MT-MMPs contain a furin recognition sequence in the C-terminus of their propeptides allowing intracellular activation.

MT1-MMP (Sato *et al.*, 1994) digests numerous substrates including collagens and several ECM proteins, as well as proteinase inhibitors, cell surface receptors and growth factors (Table I). MT1-MMP has been widely implicated in numerous physiological and pathological processes including development, promotion of cell motility and invasion, angiogenesis, cancer and rheumatoid arthritis (Holmbeck *et al.*, 2004; Itoh and Seiki, 2006). MT1-MMP can also activate gelatinase A by proteolytic removal of its prodomain (Strongin *et al.*, 1995). MT2-MMP (Will and Hinzmann, 1995) is expressed in a number of human tissues and in various forms of cancer, where its expression correlates with increased invasiveness. Like MT1-MMP, it degrades collagen I, fibrinogen and numerous other ECM proteins (Table I). MT2-MMP is also able to activate gelatinase A by a mechanism different from that of MT1-MMP (Morrison *et al.*, 2001). MT3-MMP (Takino *et al.*, 1995) is generally expressed at lower levels than MT1- and MT2-MMP. The enzyme can cleave progelatinase A, but is not involved in type I collagen invasion *in vitro* (Hotary *et al.*, 2000). Importantly,

MT1-, MT2- and MT3-MMP are the proteases that, independent of each other, confer cancer cells with the ability to degrade and invade through BMs *in vivo*, a critical event in cancer metastasis (Hotary *et al.*, 2006). MT5-MMP is predominantly expressed in the hippocampus and in cerebellum where it possibly promotes axon and dendrite extension (Llano *et al.*, 1999; Pei, 1999b).

MT4- (Puente *et al.*, 1996) and MT6-MMP (Pei, 1999c; Velasco *et al.*, 2000) differ from the other MT-MMPs in the sense that they are inefficient in cleaving progelatinase A and many ECM components. MT4-MMP has been detected in neurons in the cerebrum, smooth muscle cells and macrophages (Rikimaru *et al.*, 2007), but also in several human cancers. MT4-MMP is involved in the activation of the metalloproteinase ADMTS4 (a disintegrin-like and metalloproteinase domain with trombospondin motifs-4) *in vitro* (Gao *et al.*, 2004) and overexpression of MT4-MMP in breast cancer cells promotes primary tumor growth and metastasis *in vivo* (Chabottaux *et al.*, 2006). MT6-MMP is predominantly expressed in leukocytes but also in some forms of cancers. It promotes tumor growth and invasion of colon carcinomas *in vivo* (Sun *et al.*, 2007).

MMP-23 has a unique domain structure among the MMPs as it lacks a hemopexin domain but, instead, contains C-terminal cysteine- and proline-rich domains and a domain resembling the IL-1 type II receptor (Fig. 1) (Pei, 1999a; Velasco *et al.*, 1999). Unlike all other MMPs, MMP-23 is a type II transmembrane protein as it contains an N-terminal signal anchor (Pei *et al.*, 2000). MMP-23 is predominantly expressed in ovary, testis and prostate (Velasco *et al.*, 1999), but its functions are largely unknown.

1.2.1.6 Other MMPs

Six human MMPs do not belong to any of the groups above. Metalloelastase (MMP-12) digests elastin and numerous other ECM proteins as well as other molecules such as proTNF-α (Table I). *In vivo,* metalloelastase is involved in the generation of angiostatin (Dong *et al.* 1997; Cornelius *et al.* 1998), an inhibitor of angiogenesis that is derived by proteolytical cleavage of plasminogen. Metalloelastase is expressed primarily in macrophages and has been implicated in inflammatory skin and pulmonary diseases and cancer (Nenan *et al.*, 2005).

MMP-19 (RASI) is expressed in several normal human tissues (Cossins *et al.*, 1996; Pendas *et al.*, 1997) and can digest many ECM components *in vitro*, including various components of the BM (Table I; Stracke *et al.*, 2000). Unlike most other MMPs, it is expressed in basal keratinocytes in the healthy epidermis, but is upregulated also in the suprabasal layers in diseases such as psoriasis (Sadowski *et al.*, 2003b; Suomela *et al.*, 2003). In wounds, it is expressed in keratinocytes outside the migrating area. In skin cancers MMP-19 is expressed in hyperproliferative epidermis but not in invading cells (Impola *et al.*, 2003). MMP-19 increases cellular proliferation as well as migration and adhesion to collagen I *in vitro* through the cleavage of IGFBP-3 and subsequent release of insulin-like growth factor (IGF) (Sadowski *et al.*, 2003a). Further, MMP-19 is downregulated by the transcription factors Tst-1 and Skn-1 that promote keratinocyte differentiation and decrease proliferation (Beck *et al.*, 2007).

The expression of enamelysin (MMP-20) is highly restricted to dental tissues (Llano *et al.*, 1997). It degrades amelogenin, a major protein component of the enamel matrix and is essential for proper enamel development (Caterina *et al.*, 2002).

MMP-21 (XMMP) was originally cloned from *Xenopus laevis* and is a secreted protease with a furin recognition site (Yang *et al.*, 1997). Its human homologue is expressed in a number of fetal and adult tissues and in some carcinomas (Ahokas *et al.*, 2002). In basal and squamous cell carcinomas it is expressed in invasive cancer cells (Ahokas *et al.*, 2003).

MMP-27 was originally cloned from chicken embryonic fibroblasts (Yang and Kurkinen, 1998). Chicken MMP-27 digests casein and gelatin *in vitro*, but its mammalian homologue is poorly characterized (Clark *et al.*, 2003).

1.2.2 Targeted disruption of *Mmp* genes in the mouse

To date, at least 15 mouse models have been generated where specific *Mmp* genes have been deleted. Most of these have shown unexpectedly subtle phenotypes, and all survive until birth. It is possible that the MMPs are dispensable for embryonic development, but the explanation may also be found in enzymatic redundancy or compensation, as has been indicated by the generation of MMP double mutants (Table II; Page-McCaw *et al.*, 2007).

The generation of MT1-MMP deficient mice directed much interest towards this enzyme. This is the only *Mmp* knock-out (KO) mouse generated so far exhibiting a major phenotype, one much more severe than what have been observed by deleting genes coding for soluble *Mmp*s (Table II). The *Mt1-mmp* KO mice showed sever developmental defects in bone formation and angiogenesis, mostly due to lack of collagenolytic activity (Holmbeck *et al.*, 1999; Zhou *et al.*, 2000). Gelatinase A deficient mice, on the contrary, exhibit a mild phenotype (Itoh *et al.*, 1997) with no resemblance to the much more severe phenotype of *Mt1-mmp* deficient mice. These results indicate that collagen degradation, but not activation of progelatinase A, is a critical function of MT1-MMP *in vivo*. *Gelatinase A* and *Mt1-mmp* double KO mice, however, display severe defects in blood vessels and muscle fibers, and die immediately after birth due to respiratory failure (Oh *et al.*, 2004).

The mice deficient in gelatinases A and B, collagenase-3, enamelysin and the TIMPs have subtle phenotypes that emerge over time during the lifespan of the animals. Interestingly, these mouse models reveal that even though the MMPs are dispensable for the development of blood vessels and bone in the embryo, they are required for the postnatal development and remodeling of these tissues (Page-McCaw *et al.*, 2007).

Deletion of other *Mmp* genes has not generated obviously altered phenotypes. However, the lack of other *Mmp* genes results, in many cases, in altered reactions to specific pathogenic challenges revealing functions for these MMPs in disease progression, inflammation and regenerative processes (Fingleton, 2007). The matrilysin deficient mice, for example, fail to activate intestinal crypt α-defensins, making these mice more susceptible to bacterial infection (Wilson *et al.*, 1999). Upon lung injury, the influx of neutrophils into the alveolar space of these mice is also reduced due to lack of release of chemotactic chemokines (Li *et al.*, 2002). Further, tracheal wounds of these mice re-epithelialize more slowly. This defect is a consequence of the inability of tracheal epithelial cells to migrate over the wound area due to the lack of E-cadherin processing (McGuire *et al.*, 2003).

Table II. Phenotypic characteristics of *Mmp* and *Timp* deficient mice.

Gene	Mouse phenotype
Mmp-2	Reduced body size; reduced neovascularization; delayed mammary gland development; reduced lung saccular development; decreased allergic inflammation.
Mmp-3	Altered structure of neuromuscular junctions; delayed wound healing; hypomorphic mammary gland development.
Mmp-7	Decreased re-epithelialization after lung injury; innate immunity defects; decreased movement of neutrophils and chemokines upon injury and inflammation; reduced shedding of Fas-ligand and epithelial cell apoptosis.
Mmp-8	Increased skin tumors; delayed neutrophil recruitment to dermis surrounding skin tumors; resistance to TNF-induced lethal hepatitis.
Mmp-9	Bone-development defects; defective neuronal remyelination after nerve injury; delayed healing of bone fractures; impaired vascular remodeling; impaired angiogenesis; altered chemokine and neutrophil movement upon injury and inflammation; enhanced airway inflammation in allergic lung inflammation.
Mmp-10	Enhanced pneumonia upon bacterial infection.
Mmp-11	Delayed mammary tumorigenesis; reduced mammary carcinogenesis upon carcinogen treatment.
Mmp-12	Diminished recovery from spinal cord injury; increased angiogenesis due to decreased angiostatin; resistance to cigarette smoke-induced emphysema; reduced movement of neutrophils and chemokines upon injury and inflammation.
Mmp-13	Bone remodeling defects; reduced hepatic fibrosis; increased collagen accumulation in atherosclerotic plaques; attenuated inflammatory reaction during cholestasis.
Mmp-14	Skeletal remodeling defects; angiogenesis defects; inhibition of tooth eruption and root elongation; defects in lung and submandibular gland; inadequate collagen turn-over and early postnatal death.
Mmp-17	No detected phenotype.
Mmp-19	Obesity; early onset of tumor angiogenesis.
Mmp-20	Defects in tooth enamel.
Mmp-24	Abnormal response to sciatic nerve injury.
Mmp-2* and *Mmp-9	Complete inhibition of tumor vascularization and growth; resistance to experimental autoimmune encephalomyelitis due to lack of dystroglycan cleavage and leukocyte infiltration through the blood-brain barrier.
Mmp-2* and *Mmp-3	No detected phenotype.
Mmp-2* and *Mmp-14	Immediate postnatal death with respiratory failure, abnormal blood vessels and immature muscle fibers.
Mmp-9* and *Mmp-13	Severely impaired endochondral bone formation.
Timp-1	Accelerated endometrial gland formation; impaired learning and memory; accelerated hepatocyte cell-cycle progression; increased resistance to bacterial infection.
Timp-2	Motor defects; deficient MMP-2 activation.
Timp-3	Accelerated apoptosis in mammary gland; impaired bronchiole branching; enhanced metastatic dissemination; increased cartilage degradation with age; deficient innate immunity.

Based on Sternlicht and Werb, 2001; Oh *et al.*, 2004, Stickens *et al.*, 2004; Masson *et al.*, 2005; Agrawal *et al.*, 2006; Jost *et al.*, 2006; Kassim *et al.*, 2007; Manicone and McGuire, 2007; Page-McCaw *et al.*, 2007; Rikimaru *et al.*, 2007.

1.2.3 Epilysin (MMP-28)

Epilysin (MMP-28) is the most recently cloned member of the MMP-family. It was originally cloned from human keratinocyte and testis cDNA libraries (Lohi *et al.*, 2001), and also from lung cDNA (Marchenko and Strongin, 2001).

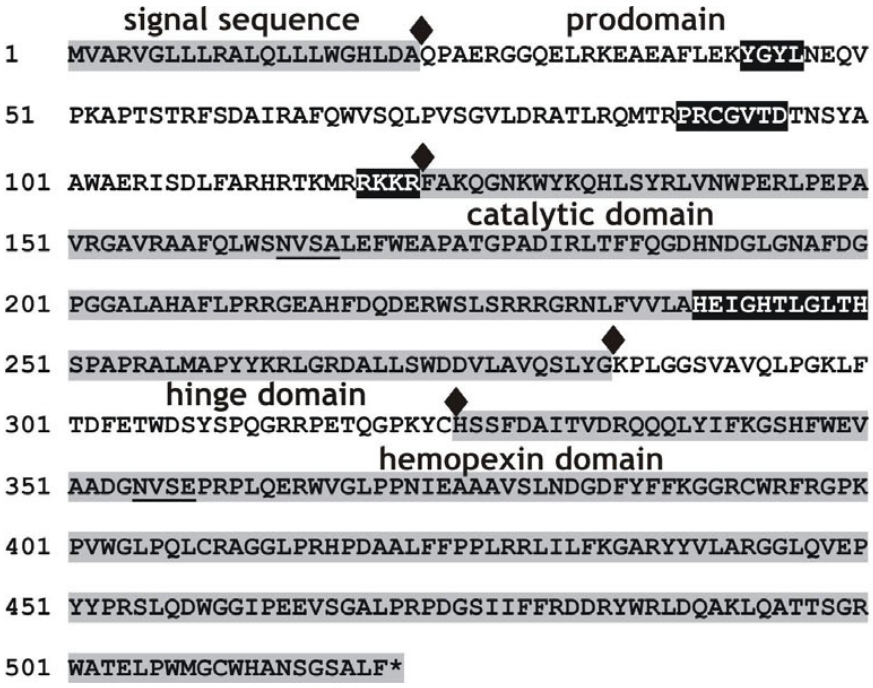


Figure 2. Amino acid sequence and domain structure of human epilysin. The amino acid sequence of epilysin is given in the one-letter code. Different protein domains are indicated above the sequence and the border between adjacent domains by ♦. The pro-sequence PRCGVTD, the zinc-binding sequence HEIGHTLGLTH and the furin-recognition sequence RKKR are indicated with a black background, as well as the conserved YGYL sequence in the prodomain. Two potential N-glycosylation sites are underlined. Numbers on the left indicate the positions of amino acids (Lohi *et al.*, 2001).

The cDNA contains an open reading frame of 1560 nucleotides coding for a 520-aa protein. The protein contains all the typical MMP domains; a hydrophobic signal sequence is followed by the prodomain including a PRCGVT-sequence. A furin recognition site RKKR is present in the C-terminal end of the prodomain. This sequence serves as a target for the furin-family of proprotein convertases that are membrane bound serine proteases, both in the trans-Golgi network and at the cell surface (Bassi *et al.*, 2005). Similar sequences are found in all the MT-MMPs but of the soluble MMPs only stromelysin-3 contains it (Pei and Weiss, 1995). The following catalytic domain is typical for soluble MMPs, but the catalytic sequence HEIGHTLGLTH is unique as no other MMP contains threonine within this sequence. A 39-aa hinge region is followed by a typical hemopexin domain (Fig. 2).

Comparison of the amino acid sequence of epilysin with the other MMPs indicates that epilysin is most closely related to another relatively new member, MMP-19 (Fig. 3, Lohi *et al.*, 2001). The *epilysin* gene is located on chromosome 17 (17q11.2), a locus that differs from all other reported *Mmp* genes (Marchenko and Strongin, 2001). *Epilysin* has a unique structural gene organization as it consists of eight exons instead of ten, which is typical for *Mmp* genes (Lohi et al., 2001). *Stromelysin-3* is the only other *Mmp* gene with eight exons

(Anglard *et al.*, 1995), but overall the organization of the *epilysin* gene most closely resembles that of *Mmp-19* (Mueller *et al.*, 2000).

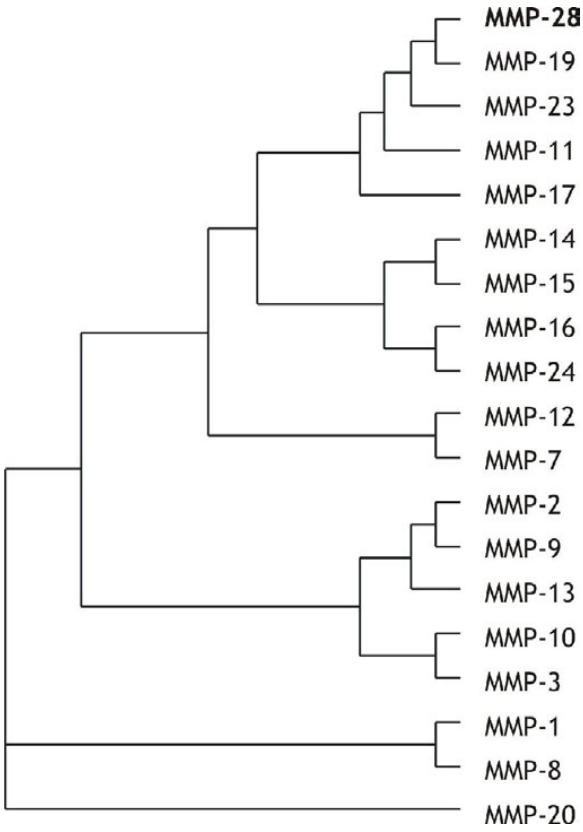


Figure 3. Dendogram of the catalytic domains of human MMPs. The amino acid sequences of the catalytic domains of human MMPs were retrieved from GenBank™ and aligned with ClustalW to generate a phylogenetic tree. Epilysin is most closely related to MMPs 19, 23, 11, and 17 (Lohi *et al.*, 2001).

Among human tissues, epilysin mRNA is highly expressed in the testis and at lower levels in the lung, heart, colon, intestine and brain (Lohi *et al.*, 2001), and also in several fetal tissues and in the adult kidney and pancreas (Marchenko and Strongin, 2001). In the rat, higher levels of epilysin mRNA were detected by real time PCR in the bone, kidney and placenta, but not in testis (Bernal *et al.*, 2005), pointing at differences in the mRNA expression between species. When specific cell types from these tissues were analyzed, epilysin expression was detected only in primary human keratinocytes grown on collagen and in transformed HaCaT keratinocytes. Epilysin protein was detected in the conditioned medium of HaCaT keratinocytes as a prominent 58 kDa band and a less prominent 55 kDa band by SDS-PAGE and immunoblotting, indicating that the epilysin mRNA codes for a secreted protein. Recombinant epilysin protein containing only the pro and catalytic domains digested casein *in vitro*. This proteolytic activity could be inhibited by both EDTA and the specific MMP-inhibitor batimastat, indicating that epilysin is a true MMP (Lohi *et al.*, 2001). The wide expression of epilysin in a number of normal tissues suggests that it may function in tissue homeostasis.

1.2.3.1 Epilysin in wound healing

Because of the initial findings of epilysin in keratinocytes, its expression has been carefully analyzed in human skin and during wound healing. The distribution of epilysin in the intact human epidermis was visualized by immunohistochemical staining, demonstrating progressively decreasing expression in the suprabasal layers. In dermal wounds, epilysin was detected in stationary keratinocytes at a distance from the wound edge, but also in migrating keratinocytes at the wound edge (Lohi *et al.*, 2001). In contrast, in another study no epilysin was detected in normal human skin. Epilysin was, however, detected in basal, proliferating keratinocytes further away from the wound edge coinciding with the area where the type IV collagen of the BM started to show an intact pattern, but not in migrating keratinocytes at the wound edge (Saarialho-Kere *et al.*, 2002). This discrepancy may reflect differences between experimental models. The results, however, suggested that epilysin could play a role in

epithelial cell proliferation or that it may be needed to restructure the newly formed BM during wound repair.

Analysis of hypertrophic burn scars revealed that mechanical compression, a method often used in the clinical treatment of this condition, markedly upregulated the expression of epilysin (Reno *et al.*, 2005).

1.2.3.2 Epilysin in cancer

Enhanced expression of epilysin has been observed in numerous different forms of cancer, but there are also several reports on the downregulation of epilysin in malignant cells. The roles of epilysin in various forms of cancer seem to vary based on tumor type and stage of the disease. Epilysin mRNA has been detected in colon adenocarcinoma cells, ovarian carcinoma cells and pancreatic adenocarcinoma cells in culture (Marchenko and Strongin, 2001). In a microarray study including 715 human proteases the expression of epilysin was strongly elevated in invasive ductal cell carcinomas (Overall *et al.*, 2004). In this study epilysin was the only MMP that was significantly upregulated in the cancer tissue. Similarly, in a real time RT-PCR study where the expression levels of all human MMPs in urothelial carcinoma extracts were examined epilysin, gelatinase A, and MT1-MMP were highly expressed (Wallard *et al.*, 2006). By laser capture microdissection of the frozen tumors, the authors localized the expression of epilysin to the stromal compartment of the tumors. Epilysin protein has also been detected in proliferating basal and suprabasal keratinocytes in grade I squamous cell carcinomas (SCC), but not in aggressive cutaneous sclerosing basal or squamous cell carcinomas of grades II and III (Saarialho-Kere *et al.*, 2002). Epilysin mRNA was also upregulated in breast carcinoma cells exhibiting increased proliferation due to overexpression of stromelysin-3 (Kasper *et al.*, 2007). In colon carcinomas, on the other hand, epilysin was downregulated in the tumor epithelium (Bister *et al.*, 2004), and no epilysin was detected in melanoma cells (Kuivanen *et al.*, 2005).

A functional role for epilysin has been described in oral squamous cell carcinomas (OSCC) (Lin *et al.*, 2006). Epilysin mRNA was markedly upregulated in OSCC samples as compared to oral premalignant lesions. Capturing epilysin mRNA in OSCC and esophageal carcinoma cell lines with antisense oligodeoxynucleotides reduced the secretion of epilysin from these cells as well as the ability of these cells to form colonies in soft agar without affecting cell growth. These results indicate a role for epilysin in the anchorage-independent growth of both OSCC and esophageal carcinomas.

1.2.3.3 Expression of epilysin in specialized tissues

Several studies imply the expression of epilysin in cartilage. For example, increased expression of epilysin mRNA was detected by real-time PCR in the cartilage of patients suffering from osteoarthritis (OA) (Kevorkian *et al.*, 2004). Similarly, epilysin and also other MMPs, were upregulated in the synovium of OA patients (Davidson *et al.*, 2006). Epilysin is, however, not very abundant in these tissues as the expression levels of collagenase-3 are approximately 20-fold higher in OA cartilage. Similar results have been reported in studies with cartilage from patients suffering from rheumatoid arthritis (Momohara *et al.*, 2004). These studies implicate that epilysin could be involved in the cartilage destruction involved in both diseases. Interestingly, histone deacetylase (HDAC) inhibitors that regulate the expression of several genes through modifying the acetylation of histones and consequently the access of transcription factors to specific genes, block cartilage destruction in an *ex vivo* model system (Young *et al.*, 2005). This repression was dependent on the decrease in the expression of several members of the MMP-family, most importantly collagenases 1 and 3.

On the contrary, other MMPs including epilysin were induced by HDAC inhibitors. The authors suggest that HDAC inhibitors could be used as selective inhibitors of proteolysis in OA, but the relevance of their ability to upregulate epilysin remained undefined. Further, the expression of epilysin, in addition to other MMPs, has been detected by real time PCR in mesenchymal stem cells, where its expression was upregulated during chondrogenesis (Djouad *et al.*, 2007).

In a study on mRNA and protein expression in human skeletal muscle, the expression level of epilysin mRNA was downregulated after 48 hours of immobilization, whereas the protein levels remained unchanged (Urso *et al.*, 2006). No significant changes in the levels of other MMPs were detected, but TIMP-1 (tissue inhibitor of MMP) was similarly affected by the procedure. The authors suggest that these results may have consequences in muscle atrophy caused by immobilization.

Further, two investigations concern the expression of epilysin in tissues related to inflammation. Epilysin protein was detected in the apical regions of the villi in normal ileum and colon, where the expression remained unaffected by inflammation (Bister *et al.*, 2004). Prominent expression of epilysin mRNA was, however, detected in T-lymphocytes where its expression was decreased upon stimulation with phorbol-12,13-myristate and ionomycin (Bar-Or *et al.*, 2003). The pro-inflammatory cytokine TNF-α has further been proposed to induce the expression of epilysin in cell culture (Saarialho-Kere *et al.* 2002).

Expression of the epilysin homologue in *Xenopus laevis* was detected in neural tissues, both in the central nervous system (CNS) and in peripheral nerves during later stages of development (Werner *et al.*, 2007). Expression of XMMP-28 was also detected in regenerating nerves during the regeneration of *Xenopus* hindlimbs (King *et al.*, 2003; Werner *et al.*, 2007), indicating that XMMP-28 serves a similar role in the neural system during development and repair. The expression of XMMP-28 was downregulated when the process of myelination was initiated both *in vivo* and *in vitro*, and *in vitro* XMMP-28 degraded the neural cell adhesion molecule NCAM. The authors speculate that XMMP-28 could alter the neuronal microenvironment leading to the development of myelin, induce a more motile phenotype in the glial cells or alter interactions between glial cells and axons by modifying cell surface structures (Werner *et al.*, 2007).

1.2.4 Regulation of MMP activity

Because of the great potential of the MMPs to degrade the ECM and remodel or even destroy tissues, their expression, activation, localization, inhibition and degradation are tightly regulated processes allowing for control on multiple levels (Fig. 4).

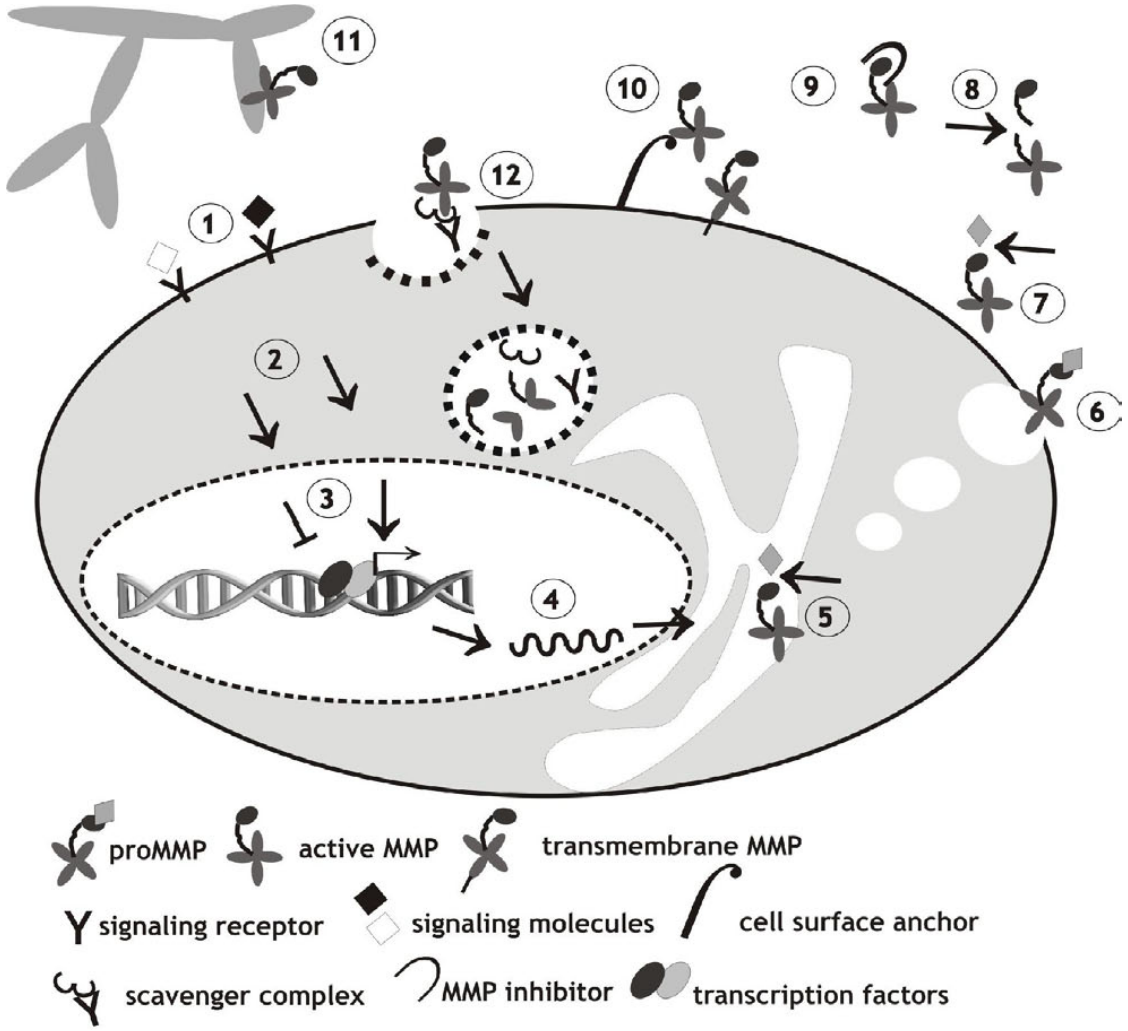


Figure 4. Multiple levels of regulation of MMP activity. 1) activating and repressive signaling; 2) intracellular signal transduction; 3) transcriptional activation and repression; 4) post-transcriptional mRNA processing; 5) intracellular activation of furin-susceptible MMPs; 6) secretion; 7) proteolytic activation; 8) proteolytic processing and degradation; 9) enzyme inhibition; 10) cell surface localization and anchoring; 11) ECM localization; 12) endocytosis and intracellular degradation. Based on Sternlicht and Werb, 2001.

1.2.4.1 Transcriptional regulation

Because of the overlapping substrate specificity of many MMPs (Table I), the biological functions of specific family members are largely determined by their individual expression patterns. These are tightly regulated at the level of gene expression by individual, unique combinations of transcriptional activators and repressors. With the exceptions of gelatinase A, matrilysin, MMP-19 and epilysin, which are constitutively expressed in normal tissues, most

MMPs are induced only in repair or remodeling processes or in response to disease or inflammation (Ra and Parks, 2007). Several of the *Mmp* promoters, however, share common elements. The promoters of the *Mmp* -1, -3, -7, -9, -10, -12, -13, -19, and -26 genes all contain a TATA-box approximately 30 bp prior to their transcription start sites and an activating protein -1 (AP-1) binding site at approximately -70 bp (Yan and Boyd, 2007). Cytokines and growth factors including interferons, interleukins, epidermal growth factor (EGF), keratinocyte growth factor (KGF), nerve growth factor (NGF), basic FGF, VEGF, platelet-derived growth factor (PDGF), TNF-α and TGF-β can induce the proto-oncogenes *c-fos* and *c-jun*. The proteins encoded by these genes heterodimerize and bind to the AP-1 sites, thus regulating the activity of these *Mmp* genes. In addition, these promoters often contain a PEA-3 (phosphoprotein enriched in astrocytes) site, which binds the ETS (E26 transformation-specific) family of oncoproteins, and cooperates with the AP-1 site in regulating the genes. The close proximity of these two regulatory sites within the promoters is crucial for the interaction. As many MMPs release active growth factors from the ECM, negative and positive feedback loops are created where the MMPs eventually regulate their own expression (Sternlicht and Werb, 2001). Coordination of the basal expression of *Mmp* genes based on similarities in their promoter structures has also been proposed, for example in the case of gelatinase A and MT1-MMP, and also their inhibitor TIMP-2 (Lohi *et al.*, 2000). The promoters of these genes all include a functional binding site for the transcriptional activator Sp1 (specificity protein 1).This is consistent with the coordinated function of these proteins in the activation of gelatinase A.

Numerous other regulatory elements have been implicated within different *Mmp* promoters, for example TGF-β inhibitory sites, AP-2, Sp3, nuclear factor κB (NF-κB) and retinoic acid response elements that are found in several *Mmp* promoters (Sternlicht and Werb, 2001). The effect of specific growth factors can, however, be divergent on different *Mmp* genes (Folgueras *et al.*, 2004). TGF-β, for example, suppresses the expression of collagenase-1 and stromelysin-1 but induces the expression of collagenase-3 (Uria *et al.*, 1998) and stromelysin-2 (Wilkins-Port and Higgins, 2007). The effects can also be cell type specific as for example phorbol esters preferentially induce the expression of *stromelysin-1* in fibroblasts but of *stromelysin-2* in keratinocytes (Windsor *et al.*, 1993).

Promoter polymorphisms that affect gene regulation have been identified in some *Mmp* genes (Ye, 2000). For example, single-nucleotide polymorphisms (SNPs) within the *stromelysin-1*, *gelatinase B* and *metalloelastase* promoters have been described to increase the susceptibility to coronary artery disease. Further, a SNP in the *collagenase-1* promoter has been associated with increased promoter activity and increased susceptibility to lung cancer. Conversely, a SNP in the *stromelysin-1* promoter has been associated with decreased promoter activity and decreased susceptibility to lung cancer (Yan and Boyd, 2007). Recently, the epigenetic regulation of *Mmp* gene expression has been carefully investigated and there is now evidence for the contribution of both methylation and histone acetylation in the regulation of *Mmp* gene expression (Clark *et al.*, 2007). Post-transcriptional regulation, mainly regulation of mRNA stability, has also been observed (Yan and Boyd, 2007).

1.2.4.2 Activation of latent MMPs

All MMPs are translated as inactive precursors, and activation of the latent enzymes by removal of the prodomain is another important point of regulation of their catalytic activity. This can be achieved by numerous mechanisms *in vitro*, but only a few relevant *in vivo* processes activating specific MMPs have been described (Ra and Parks, 2007). All MT-MMPs, stromelysin-3 and epilysin, contain an RXKR sequence at the C-terminal end of their prodomains that serves as a target for the furin-family of proprotein convertases (Bassi *et al.*,

2005). These MMPs are thus intracellularly activated by proteolytic removal of their prodomains during the secretory pathway.

The activation of other MMPs is achieved through proteolytic processing by other proteases. Several MMPs can activate other MMPs *in vitro* by cleaving their prodomains (Table I). The relevance of this mode of activation *in vivo* is not, however, certain as coexpression of activating MMPs and their potential MMP substrates in tissues and colocalization in the pericellular space in many cases are not evident. For example, stromelysin-1 efficiently activates procollagenase-1 and promatrilysin *in vitro*, but *in vivo* these proteases are not typically coexpressed (Ra and Parks, 2007).

The best described example of an MMP activating another MMP is the activation of gelatinase A on the cell surface by MT1-MMP. This activation is achieved through an intricate mechanism including the formation of a complex of gelatinase A, MT1-MMP and TIMP-2 (Strongin *et al.*, 1995). This process is dependent on homo-oligomerization of MT1-MMP molecules through interactions between the hemopexin and cytoplasmic domains of adjacent molecules (Itoh *et al.*, 2001; Lehti *et al.*, 2002). Plasmin and other serine proteases have also been implicated in the activation of proMMPs (Monea *et al.*, 2002; Ra and Parks, 2007). Pro-MMPs can further be subjected to so called allosteric activation. This is achieved through interactions between pro-MMPs and other molecules that induce a conformational change in the pro-MMP disrupting the cysteine-zinc interaction and allowing autolytic cleavage of the prodomain. Oxidants generated by leukocytes and other cell types are an example of molecules that can both activate and inactivate MMPs by modifying critical amino acids via oxidation. Several proMMPs are activated *in vitro* by reactive oxygen species (ROS) but their role has not been confirmed *in vivo* (Fu *et al.*, 2007).

In all situations, however, the removal of the prodomain does not seem to be obligatory for MMP activation as long as the interaction between the conserved cysteine residue in the prodomain and the active site zinc ion is disrupted (Fu *et al.*, 2007). In epidermal wound healing, for example, the catalytic activity of collagenase-1 is required for keratinocyte migration on type I collagen (Pilcher *et al.*, 1997) but in tissues only the pro-form of this enzyme has been detected (Dumin *et al.*, 2001). In this setting procollagenase-1 binds to the $\alpha_2\beta_1$ integrin, which functions as a collagen receptor. The $\alpha_2\beta_1$ integrin possibly induces an active conformation of procollagenase-1 without the removal of the prodomain and only in the presence of collagen I. Thus, $\alpha_2\beta_1$ integrin would serve to target collagenase-1 to the basal surface of keratinocytes and induce its activity transiently when in contact with its preferred substrate (Fu *et al.*, 2007).

It has also been questioned whether removal of the prodomain of MT1-MMP is needed for its catalytic activity (Cao *et al.*, 1996; 1998). Its prodomain has instead been suggested to serve as a chaperone regulating the folding and trafficking of the enzyme (Cao *et al.*, 2000), a function dependent on a conserved YGYL sequence (Pavlaki *et al.*, 2002). This sequence is found in the prodomains of all MT-MMPs but among the secreted MMPs only in MMPs 19 (Cossins *et al.*, 1996; Pendas *et al.*, 1997) and 28 (Lohi *et al.*, 2001).

1.2.4.3 Pericellular localization

Compartmentalization is an important aspect in the regulation of MMP activity. MMPs are not randomly released to the extracellular compartment but instead there are specific mechanisms that confine and concentrate proteinases in the immediate pericellular environment, targeting their catalytic activity to specific substrates in this location (Ra and Parks, 2007). In biological situations the high ratio of proteinase inhibitors to active proteinases protects the tissues from degradation. Therefore the ability to generate focused proteolytic activity in the pericellular space is crucial to locally overcome the molar excess of

inhibitors and allow for cell movement (Sternlicht and Werb, 2001). Integration of the MT-MMPs into the cell membrane is crucial for their ability to promote cell invasion (Hotary *et al.*, 2000) and several of the soluble MMPs are also confined to the pericellular environment through interactions with specific cell surface receptors (Fig. 5). For example, gelatinase A binds to the $\alpha_v\beta_3$ integrin (Brooks *et al.*, 1996), gelatinase B to CD44 (Yu and Stamenkovic, 1999) and collagenase-1 to the $\alpha_2\beta_1$ integrin (Dumin *et al.*, 2001; Stricker *et al.*, 2001). Matrilysin in turn, binds to heparan sulfate (Yu and Woessner, 2000; Yu *et al.*, 2002), cholesterol (Yamamoto *et al.*, 2006) and the tetraspanin CD151 (Shiomi *et al.*, 2005). Of these cell surface molecules, at least CD44 and $\alpha_2\beta_1$ integrin have also been proposed to mediate MMP activation and to interact with specific substrate molecules, hence greatly favoring proteolysis. TIMP-2 can simultaneously bind to the hemopexin domain of progelatinase A and the catalytic domain of MT1-MMP. Progelatinase A can be activated in this complex by another, adjacent MT1-MMP molecule, thus both sequestering gelatinase A to the cell surface and inducing its catalytic activity in this location.

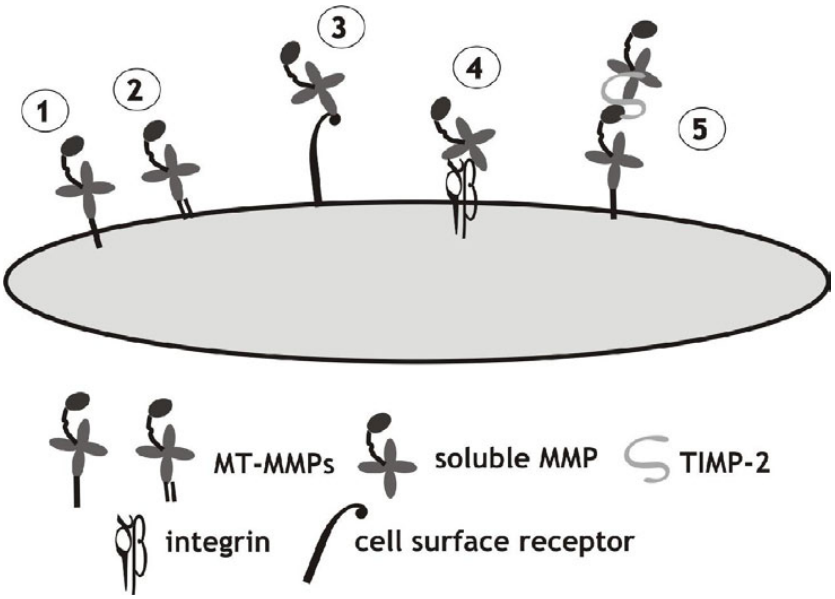


Figure 5. Localization of MMPs at the cell surface: The MT-MMPs are attached to the cell surface by single transmembrane and cytoplasmic domains (1), or by GPI-anchors (2). Soluble MMPs bind to cell surface molecules such as CD44, heparan sulfates (3) or integrins (4). Gelatinase A binds to MT1-MMP on the cell surface through TIMP-2 (5).

In contrast to the general perception of MMPs, an alternatively spliced form of stromelysin-3 can be translated as an active, intracellular proteinase (Luo *et al.*, 2002). Other MMPs with reported intracellular functions are collagenase-1, which associates with mitochondria and confers resistance to apoptosis (Limb *et al.*, 2005) and gelatinase A, which digests troponin I contributing to myocardial dysfunction upon ischemia (Wang *et al.*, 2002). Further, MMP-26 degrades the intracellular part of the estrogen receptor β delaying cancer progression in hormone sensitive breast carcinomas (Savinov *et al.*, 2006) whereas MT1-MMP can promote chromatin instability by degrading the centrosomal protein pericentrin (Golubkov *et al.*, 2005). Mislocalization of MT1-MMP in cancer thus contributes to pathogenesis by allowing interactions between this proteases and normally unavailable intracellular substrates, which further emphasizes the importance of proper compartmentalization (Strongin, 2006).

1.2.4.4 Inhibition of MMP activity

The catalytic activity of MMPs is also regulated by endogenous inhibitors. The major inhibitor in tissue fluids is the abundant plasma protein α2-macroglobulin, which forms complexes with MMPs that then bind to scavenger receptors and are irreversibly cleared by endocytosis. Thrombospondins function similarly in complex with certain MMPs (Egeblad and Werb, 2002).

The tissue inhibitors of MMPs (TIMPs) are secreted proteins that specifically and reversibly inhibit MMPs (Lambert *et al.*, 2004). In contrast to α2-macroglobulin, the four TIMPs act more locally and their tissue expression and affinity towards individual MMPs differ. The balance between MMPs and TIMPs is an important factor regulating ECM turnover and remodeling in healthy tissues, and disruption of this balance has been implicated in processes like angiogenesis and cancer invasion. In addition, TIMPs have biological effects on cell growth and survival that cannot always be explained by their ability to inhibit MMP activity (Table II). TIMP-1 was first identified as an erythroid potentiating agent, a feature shared by TIMP-2. TIMP-1 can confer resistance to apoptosis whereas TIMPs 2 and 3 promote cell death (Lambert *et al.*, 2004). The TIMPs further affect cell signaling through interactions with cell surface receptors such as $\alpha_3\beta_1$ and VEGF receptor-2 (Chirco *et al.*, 2006).

The membrane anchored glycoprotein RECK (reversion-inducing cysteine-rich protein with Kazal motifs) is a different type of MMP inhibitor. It inhibits the catalytic activity of at least gelatinases A and B and MT1-MMP, and is a potent inhibitor of tumor invasion and metastasis (Oh *et al.*, 2001). RECK is expressed in most human tissues and nonmalignant cells, whereas its expression is repressed in most cancer cells (Takahashi *et al.*, 1998). RECK has probably other functions aside from blocking MMP activity as disruption of the gene in mice is embryonic lethal (Oh *et al.*, 2001).

1.2.5 Modulation of bioactive molecules by MMPs

MMPs can degrade most components of the ECM, at least *in vitro* (Table I). The biological actions of individual MMPs are, however, much more restricted as the expression and activation of MMPs are strictly regulated in both time and space (Parks *et al.*, 2004). The identification of *in vivo* substrates for MMPs contributes critically to the understanding of their functions in both development and disease. Technologies applied to identify the biological functions of MMPs include the generation of genetically modified mice where specific MMPs are either overexpressed or abrogated (Table II, Overall and Blobel, 2007). An emerging view of MMP biology is that matrix degradation is not their sole function, as they react to challenges like injury, inflammation or cancer by releasing specific signaling molecules, such as cryptic fragments of ECM molecules with biological functions, growth factors and cell-adhesion molecules in the pericellular microenvironment (Parks *et al.*, 2004).

1.2.5.1 Cryptic fragments of ECM molecules

Cleavage of ECM molecules by MMPs frequently generates fragments that display biological functions different from their precursors or reveal biologically relevant cryptic epitopes within ECM molecules. For example, both gelatinases A and B can promote angiogenesis by exposing a cryptic epitope within collagen IV, but MMPs also release fragments of ECM molecules that are anti-angiogenic (Mott and Werb, 2004). For example, gelatinase B can release a fragment from collagen IV that has been named tumstatin. Decreased amounts of circulating tumstatin is accompanied by accelerated tumor growth in gelatinase B deficient mice, emphasizing the biological relevance of this fragment (Hamano *et al.*, 2003).

Angiostatin is an anti-angiogenic fragment that is cleaved from plasminogen by metalloelastase (Dong *et al.*, 1997; Cornelius *et al*. 1998), matrilysin and gelatinase B *in vivo* (Pozzi *et al.*, 2000). Further, the cleavage of the laiminin-5 γ2 chain by MMPs releases a fragment that induces epithelial cell migration. Mice deficient in MT1-MMP have reduced amounts of this fragment in their tissues indicating that this cleavage occurs *in vivo* and possibly affects epithelial tissue organization (Koshikawa *et al.*, 2004).

1.2.5.2 Growth factors and cytokines

The ECM functions as a reservoir for latent growth factors like TGF-β and VEGF that can be released and activated by proteolysis. In the ECM the mature form of the multifunctional TGF-β is associated noncovalently with its propeptide (latency-associated peptide, LAP) and to LTPB (Saharinen *et al.*, 1999; Hyytiäinen *et al.*, 2004). A pool of inactive TGF-β is hence maintained in the ECM from where the mature molecule can be activated when needed. Several MMPs directly activate TGF-β via cleavage of the LAP complex *in vitro* (Yu and Stamenkovic, 2000; D'Angelo *et al.*, 2001; Karsdal *et al.*, 2002), but *in vivo* this function has been associated only with MT1-MMP (Mu *et al.*, 2002) and gelatinase A (Ge and Greenspan, 2006). VEGF is a central factor in angiogenesis that stimulates the growth of new vessels. It binds heparan sulfate proteoglycans in the ECM. Gelatinase B has been implicated in the release of VEGF from the ECM in different tumor models, where angiogenesis is a crucial factor regulating tumor growth (Mott and Werb, 2004). MT1-MMP likewise upregulates the expression of VEGF in tumor cells (Deryugina *et al.*, 2002). MMPs have also been implicated in the cleavage of IGFBPs and perlecan leading to the release of IGFs and FGFs, respectively.

MMPs also act on growth factor receptors, for example by releasing the extracellular domains of these receptors into the ECM, where they may sequester growth factors and interfere with their binding to cell surface attached receptors (Vu and Werb, 2000). Further, MT1-MMP associates with the PDGF receptor β on the membrane of vascular smooth muscle cells promoting PDGF-B-induced signaling via the receptor. Through this complex MT1-MMP can affect vessel wall architecture *in vivo* (Lehti *et al.*, 2005).

Chemokines are chemotactic molecules that attract cells of the immune system to sites of injury or infection. MMPs have been implicated in the regulation of chemokine activity through direct cleavage of these molecules, thus enhancing, repressing or antagonizing their activities. MMPs can thus be both pro and anti-inflammatory agents (Page-McCaw *et al.*, 2007). The effects of MMPs on chemokines can also be indirect as they modulate other proteins that bind the chemokines to concentrate them in specific locations. In this way the MMPs are involved in the generation of chemokine gradients guiding migrating leukocytes to target sites (Parks *et al.*, 2004; Manicone and McGuire, 2007).

1.2.5.3 Cell adhesion molecules

Cell adhesion molecules are also important MMP substrates involved in numerous biological processes. E-cadherin is a transmembrane cell-cell adhesion glycoprotein localized at adherence junctions between epithelial cells. It contains a cytoplasmic tail by which it is connected to the actin microfilaments of the cytoskeleton via β- and α-catenin. E-cadherin is cleaved by stromelysin-1 and matrilysin in cultured epithelial cells (Noë *et al.*, 2001) and by gelatinase B in ovarian cancer cells (Symowicz *et al.*, 2007) This cleavage results in the release of a soluble E-cadherin fragment that functions in a paracrine fashion inhibiting E-cadherin mediated cell aggregation. This fragment further induces invasion via the upregulation of gelatinases A and B and MT1-MMP (Nawrocki-Raby *et al.*, 2003). Further, MT1-MMP can mediate the shedding of both E- and N-cadherins in a kidney ischemia model

(Covington *et al.*, 2006). Matrilysin mediates the shedding of E-cadherin from injured lung epithelium *in vivo*, and matrilysin deficient mice show decreased epithelial cell migration after injury (McGuire *et al.*, 2003).

The cell adhesion molecule CD44 is a cell surface glycoprotein involved in numerous ways in cell-cell and cell-ECM interactions, which also functions as a cell surface receptor for gelatinase B. CD44 is shed by MT1-MMP, leading to increased cell migration (Kajita *et al.*, 2001). Another cell adhesion molecule that is cleaved by MMPs is syndecan-1, which is an abundant cell surface heparan sulfate proteoglycan on epithelial cells, mediating interactions between cells and several ECM molecules. Cleavage of syndecan-1 by MT1-MMP stimulates cell migration (Endo *et al.*, 2003). Further, matrilysin mediates shedding of syndecan-1 in complex with the keratinocyte-derived chemokine KC (also known as CXCL1 in the mouse and as GROα in humans) from the mucosa of injured lungs *in vivo*. This process is necessary for directing neutrophil influx to the site of injury (Li *et al.*, 2002). MT1-MMP also interacts with and cleaves the intracellular adhesion molecule-1 (ICAM-1), thus favoring transmigration of leukocytes through the endothelial cell layer (Sithu *et al.*, 2007).

1.3 Epithelial to mesenchymal transition

Epithelial cells form uniform layers where regularly spaced cell-cell junctions and adhesion between neighboring cells hold them tightly together and restrain the movement of individual cells away from the layer. Epithelial cell layers surround organs and line cavities in the organism providing them with structural support and serving as a surface for interactions between the underlying stromal tissue and the outside. The epithelial cell layer is polarized. The basal and apical surfaces express different surface molecules and serve different functions. Mesenchymal cells, in contrast, form irregular structures with weak intracellular adhesion, which facilitates migration. Mesenchymal cells have a more elongated shape and are able to move individually, whereas epithelial cells in general move as a sheet (Lee *et al.*, 2006).

Epithelial to mesenchymal transition (EMT) is a fundamental biological process where epithelial cells acquire a more mesenchymal phenotype, including the loss of polarity and the adoption of a morphology appropriate for migration and invasion. Hallmarks of EMT are loss of cell-cell adhesion receptors, loss of cell-matrix contacts and changes in the composition of the intermediate filament cytoskeleton. EMT is a critical process in the induction of cell movement and provides stationary cells with the means to invade into surrounding tissues. EMT is therefore essential in embryonic development, but it can also be induced in adult tissues, which contributes to the development and progression of pathological processes such as fibrosis and cancer (Thiery, 2002; Huber *et al.*, 2005). EMT can be induced through different molecular pathways by combining the actions of locally expressed growth factors with proteolytic degradation of critical targets. These targets comprise the BMs on which the epithelial cells reside and adhesion molecules holding the epithelial sheet intact (Kalluri and Neilson, 2003). Degradation of E-cadherin is a crucial event involved in EMT, invasive tumor growth and metastasis (Bissell and Radisky, 2001; Tse and Kalluri, 2007). As noted above (section 1.2.5.3) several MMPs are able to cleave the extracellular part of E-cadherin, and as a result of this contribute to the induction and progression of EMT. MMPs are, however, also involved in several other molecular pathways, which contribute to the transformation of epithelial cells (Fig. 6, Egeblad and Werb, 2002; Page-McCaw *et al.*, 2007; Przybylo and Radisky 2007).

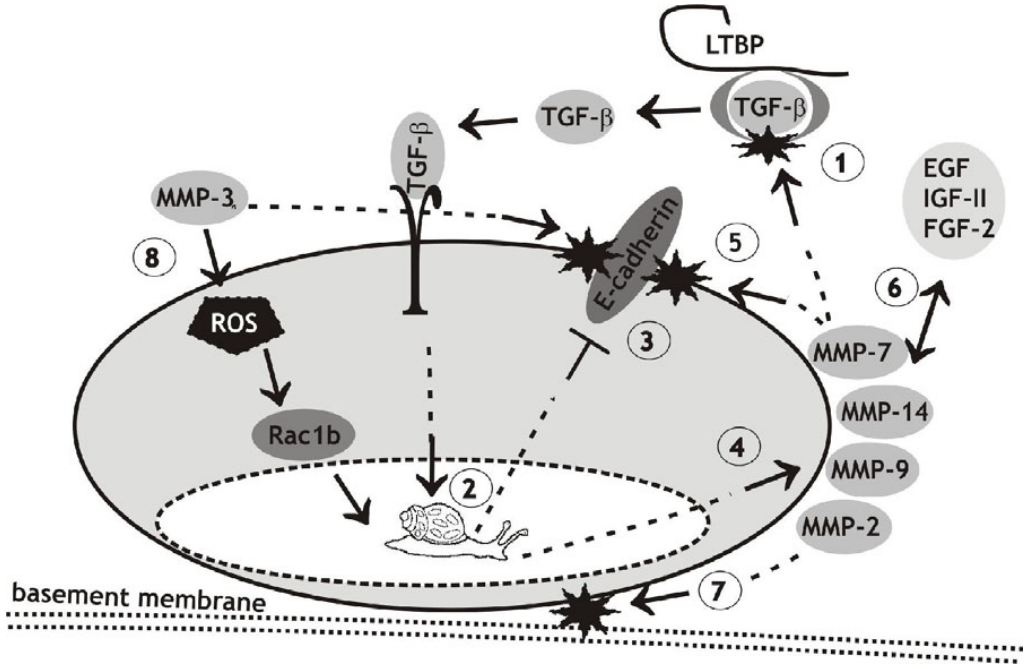


Figure 6: MMPs affect EMT. MMPs can contribute to EMT by different actions. MMPs release and activate ECM-bound latent TGF-β (1). TGF-β is a potent inducer of EMT, and its main target in this process is the transcriptional regulator Snail (2). Snail downregulates the expression of the cell surface adhesion receptor E-cadherin (3), the loss of which is an important event in the induction of EMT. Snail also induces the expression of certain *Mmp* genes (4). MMPs may further induce EMT through the shedding of the extracellular part of E-cadherin (5). MMPs also release other growth factors from the ECM that facilitate the induction of EMT and also regulate the expression of the MMPs themselves (6). Some MMPs participate in the degradation of the BM (7), another action contributing to EMT. Stromelysin-1 (MMP-3) can induce EMT through the degradation of E-cadherin (3), but also through the induction of Snail via Rac1b and the release of intracellular ROS (8).

1.3.1 Role of MMPs in growth factor mediated EMT

Induction of EMT is facilitated by local expression of TGF-β, EGF, IGF-II or FGF-2 (Thiery, 2002), all of which can be released from the ECM through the actions of MMPs (Vu and Werb, 2000). The ability of TGF-β to inhibit the growth of normal cells is well known (Massague and Gomis, 2006). Nevertheless, members of the TGF-β family can induce and maintain EMT in a variety of biological and pathological processes like cancer and fibrosis (Miettinen *et al.*, 1994; Zavadil and Böttinger, 2005). TGF-β thus plays a dual role in cancer as it promotes the acquisition of an invasive phenotype in tumor cells that have become resistant to its growth inhibitory effects (Moustakas *et al.*, 2002). Little is known of the complex events underlying the conversion of TGF-β from a growth inhibiting to a tumor promoting growth factor. It is possible, that a change in the repertoire of available intracellular signal mediators shifts the TGF-β response from favoring growth inhibition to promoting EMT as cancer progresses (Tse and Kalluri, 2007).

EMT can be initiated by autocrine and paracrine TGF-β activation of major signaling pathways fundamentally altering the transcriptional activity of epithelial cells (Zavadil *et al.*, 2001). TGF-β activates several signaling pathways including the Smad-, Ras-, mitogen-activated protein kinase- (MAPK), WNT-, NF-κB- and phosphatidylinositol 3 kinase- (PI3K) pathways. Of these the Smad pathway seems to control a majority of TGF-β target genes during EMT (Valcourt *et al.*, 2005). Smad-3 plays a crucial role in several *in vivo* models of EMT (Roberts *et al.*, 2006), whereas Smad-2 has been pointed out as a critical mediator of TGF-β-induced EMT in cancer progression (discussed in Lee *et al.*, 2006). Other signaling

molecules and pathways, or in many cases cross-talk between different pathways, seem to play key roles in other tissues and under different circumstances (Huber *et al.*, 2005). The role and effects of TGF-β in EMT is therefore highly dependent on the cellular context (Zavadil and Böttinger, 2005; Peinado *et al.*, 2007).

TGF-β, as well as FGF-2, further contribute to the induction of EMT by increasing the synthesis and activation of gelatinases A and B (Strutz *et al.*, 2002; Li *et al.*, 2003b; Van Themsche *et al.*, 2007). This has been proposed to lead to degradation of the collagen IV component of the BM, a central process in increasing epithelial cell plasticity. Degradation of collagen IV by gelatinase A was pointed out as an important event the TGF-β-induced EMT in avian heart development (Song *et al.*, 2000). However, the expression of MT1-MMP was also increased in this study. This is an important notion as MT1-, MT2-, and MT3-MMP have been proposed as the only proteases involved in BM degradation *in vivo* (Hotary *et al.*, 2006). Other observations support a role for gelatinase A only in the migratory phase in EMT in the avian embryo (Duong and Erickson, 2004). In addition to the gelatinases, also collagenase-3 and MT1-MMP are frequently upregulated in EMT to drive the invasion of the phenotypically altered cells through BMs and into surrounding tissues (Murphy and Gavrilovic, 1999; Seiki and Yana, 2003), further emphasizing the role of MMPs in carcinogenesis. Conversely, these MMPs can activate TGF-β in osteoblasts, chondrocytes, and epithelial cells (Yu and Stamenkovic, 2000; D'Angelo *et al.*, 2001; Karsdal *et al.*, 2002; Mu *et al.*, 2002). In SCC cells TGF-β induces the expression of collagenases 1 and 3 and enhances cell invasion via Smad signaling (Johansson *et al.*, 2000; Leivonen *et al.*, 2006). In other circumstances, TGF-β downregulates collagenase-1 and stromelysin-1 (Uria *et al.*, 1998), pointing out the complex relationship between TGF-β and MMP function in the induction and progression of EMT and cancer (Derynck *et al.*, 2001).

1.3.2 MMPs are induced by Snail and Sip1

A major target for TGF-β in EMT is the transcription factor Snail that is induced through the MAPK, PI3K and Smad pathways (Peinado *et al.*, 2003, Huber *et al.*, 2005). Snail is a strong repressor of E-cadherin expression (Batlle *et al.*, 2000; Cano *et al.*, 2000), and hence a potent inducer of EMT. TGF-β can coordinate the expression and function of Snail with respect to other transcriptional repressors, such as members of the ZEB and basic helix-loop-helix (bHLH) families that are involved in the induction of EMT and the maintenance of the migratory phenotype (Peinado *et al.*, 2007). In addition to E-cadherin, Snail acts on numerous other target molecules including the repression of other proteins involved in cell adhesion, such as claudin and occludin (De Craene *et al.*, 2005). In parallel, Snail induces the production of mesenchymal marker molecules such as vimentin and fibronectin that facilitate the movement of the transformed epithelial cells, and also of ECM remodeling enzymes, such as the MMPs (Fig. 6). *In vitro* Snail has been proposed to induce the expression of gelatinase A, matrilysin and MT1-MMP (Yokoyama *et al.*, 2003; Miyoshi *et al.*, 2004), but the interactions between Snail and the promoters of these genes are not understood in detail at present. When overexpressed in Madin-Darby canine kidney (MDCK) cells, Snail induces expression of gelatinase B as part of the EMT process (Jorda *et al.*, 2005). Snail does not seem to interact directly with the *gelatinase B* promoter, but the binding of Sp1 to a specific GC-box within the promoter is required for induction. Conversely, silencing of Snail by RNA interference in carcinoma cells decreased the levels of gelatinase B and angiogenic markers, dramatically reducing *in vivo* tumor growth and invasion (Olmeda *et al.*, 2007).

Sip1 (Smad-interacting protein-1) is another transcriptional repressor of E-cadherin (Comijn *et al.*, 2001) which is activated in mouse mammary epithelial cells undergoing EMT in response to TGF-β (Thiery, 2002). Sip1 can affect gene regulation either independently or

in concert with Snail, which possibly regulates the expression of Sip1 itself (Takkunen *et al.*, 2006). The Snail and Sip1 binding sites within the E-cadherin promoter are overlapping, and these two factors are often coexpressed in carcinomas negative for E-cadherin. *In vitro*, Sip1 can induce the expression of collagenase-1, gelatinase A and MT1-MMP (Miyoshi *et al.*, 2004), but the relevance of this induction *in vivo* remains unclear. Increased expression of both Sip1 and Snail has, however, been observed in several human cancers (Peinado *et al.*, 2007) like breast and ovarian carcinomas, where high levels of Sip1 has been correlated with poor prognosis (Elloul *et al.*, 2005).

A third important transcription factor repressing the *E-cadherin* gene and inducing EMT is Twist, a protein known to be essential during development (Yang *et al.*, 2006). Twist is upregulated early during metastatic progression and has functionally been validated as a critical regulator of lung metastasis is mammary tumor models (Yang *et al.*, 2004).

1.3.3 MMPs induce Snail

Overexpression of stromelysin-1 in mammary epithelial cells can cause stable EMT, accompanied by the cleavage of E-cadherin, upregulation of endogenous MMPs and increased invasiveness (Lochter *et al.*, 1997). Further, when these stromelysin-1 overexpressing cells were injected into mice, they formed invasive tumors, indicating that stromelysin-1 can trigger EMT and render cells tumorigenic and invasive also *in vivo* (Sternlicht *et al.*, 1999). Both *in vivo* and *in vitro*, stromelysin-1 activity was needed only for the induction of EMT but not for sustaining the converted phenotype. The stromelysin-1-induced EMT is mediated by Rac1b, an alternatively spliced form of the small GTPase Rac1, which increases the levels of intracellular ROS, leading in turn to increased expression of Snail and EMT (Fig. 6, Radisky *et al.*, 2005).

In conclusion, MMPs play multiple roles in the process of EMT, both as inducers and effectors of epithelial transformation, participating in several central regulatory pathways and molecular feedback circuits.

1.4 MMPs in cancer

The functions of MMPs in cancer are not restricted to their role in the induction and maintenance of the transformed epithelial phenotype. The expression and activation of MMPs is increased in almost all human cancers where they regulate central processes like cancer cell growth, differentiation, apoptosis, migration and invasion, and regulation of tumor angiogenesis and function of the immune system. Increased expression of MMPs often correlates with poor prognosis (Folgueras *et al.*, 2004). Some MMPs, such as matrilysin, are generally produced by cancer cells themselves, whereas others such as the gelatinases are produced by tumor stromal cells, namely fibroblasts, inflammatory cells and endothelial cells. MMPs exert their effects on cancer progression by cleaving a wide set of target proteins, including both structural ECM components and also growth factor binding proteins, their receptors and precursors, cell adhesion molecules and cell surface receptors (Westermarck and Kähäri, 1999; Egeblad and Werb, 2002). Much of the evidence for the active role of MMPs in cancer stems from animal studies, either concerning the growth of transplanted MMP or TIMP-expressing cancer cells in mice, or from studies of cancer induction and progression in genetically modified mice lacking or overexpressing specific MMPs or TIMPs (Table II; Egeblad and Werb, 2002).

Because of the fundamental role of MMPs in cancer, the possibility to use MMP inhibitors in anti-cancer therapy has been much investigated. Attempts have been made to inhibit MMP synthesis, to inhibit interactions between MMPs and other proteins, to exploit

the increased MMP activity to generate targeted toxin release and to block MMP activity (Egeblad and Werb, 2002). The results have, however, been discouraging and none of the developed drugs have so far passed clinical trials (Fingleton, 2007). Broad-spectrum inhibitors have caused adverse and unforeseen side effects, revealing the critical need for inhibitors specifically targeting individual cells and MMPs. Thorough investigations on the biological functions of the MMPs, the redundancy and cross-talk between different family members, and different aspects of regulating their activity are still needed (Overall and Kleifeld, 2006; Fingleton, 2007). There is an increasing amount of evidence suggesting that the expression of certain MMPs provides beneficial and protective effects in several stages of cancer progression. This is an additional aspect that should be taken into consideration when designing MMP inhibitors for clinical use (Martin and Matrisian, 2007). These observations may also partly explain the failure of many current MMP inhibitors in clinical trials.

1.5 MMPs and other proteolytic pathways

In addition to the MMPs there are several other important protease families participating in the remodeling of the ECM. These different protease systems frequently interact on the cell surface, generating proteolytic cascades accurately fine-tuning cell signaling and phenotype according to the conditions of the cellular microenvironment (Sternlicht and Werb, 2001). ADAMs (a disintegrin-like and metalloproteinase domain) are transmembrane cell surface metalloproteinases that are involved in the shedding of the extracellular domains of several membrane-bound proteins. ADAMs activate several growth factors and cytokines and regulate signaling pathways involved in various processes from development to cancer progression (Huovila *et al.*, 2005). For example ADAM-17 (TNF-α converting enzyme, TACE) cleaves and activates membrane-bound TNF-α *in vivo* (Peschon *et al.*, 1998). The ADAMs also contain a disintegrin-like domain through which individual family members can participate in cell adhesion via interactions with integrins. The ADAMTS (ADAM with trombospondin motifs) metalloproteinases are secreted enzymes that contain at least one trombospondin type I repeat. They are involved in the processing of procollagens and other ECM proteins such as aggrecan, and play important roles in processes like ECM organization, inflammation and cell migration (Apte, 2004). The activity of the ADAMs is regulated by the TIMPs, and like MMPs, the affinity of specific TIMPs for specific ADAMs is individual. Only TIMP-3 can inhibit the ADAMTS proteases (Kashiwagi *et al.*, 2001).

Serine proteases, such as the members of the plasmin activation system, are also important mediators of pericellular proteolysis and ECM remodeling. Plasmin is primarily involved in the proteolysis of the fibrin clot in wound healing, but can also degrade various other ECM components. The involvement of the urokinase-type plasminogen activator (uPA) in cell migration, invasion and tissue remodeling is enabled by its specific targeting to cell membranes through interactions with the uPA receptor (uPAR) (Qiu *et al.*, 2007). Through the activation of plasminogen, and also due to its intrinsic catalytic activity, uPA can degrade various ECM components and activate growth factors, such as hepatocyte growth factor (HGF) and TGF-β (Rifkin *et al.*, 1999). Importantly, uPA and plasmin are also involved in the activation of other proteases *in vivo*, such as collagenase-2, stromelysin-1 and gelatinase B (Andreasen *et al.*, 2000). In this way, proteolytic activity can be amplified at the cell surface to locally overcome the otherwise excess of inhibitors in tissues facilitating cell movement and ECM remodeling.

2 Outline of the present study

When this study was initiated in 2000, epilysin had recently been cloned and initial characterization carried out (Lohi *et al.*, 2001). Epilysin was defined as an epithelial MMP expressed in many human tissues unlike most other MMPs. The aim of the present work was to elucidate the biological functions of this new MMP and to reveal its possible involvement in pathological processes. For this purpose we generated stable cell pools overexpressing recombinant epilysin, as the protease is expressed at low levels in cultured cells. During the course of the study, we unexpectedly found that overexpression of epilysin causes epithelial to mesenchymal transition in human lung carcinoma cells through mechanisms dependent on TGF-β.

The aims of the work were:

1. to analyze how the expression of the epilysin gene is regulated through its promoter region and to identify transcriptional regulators that interact with it. For this purpose both the human and mouse promoters were sequenced and compared with each other to identify conserved promoter regions potentially important in gene regulation.
2. to characterize the biochemical properties of epilysin, in particular to identify potential substrates and other interacting proteins affecting the activation, localization and inhibition of epilysin.
3. to clone the cDNA of the mouse epilysin homologue and compare it with the known human cDNA.
4. to define the expression of epilysin in different tissues in the mouse and to explore what physiological functions it could participate in and what kind of substrates it could encounter *in vivo.*
5. to analyze how overexpression of epilysin affects the properties of cultured epithelial cells.

3 Materials and Methods

3.1 Cell culture and reagents

All cells were grown in Dulbecco's modified Eagle's medium (D-MEM) or Eagle's minimal essential medium (MEM) containing 10 % (v/v) heat inactivated fetal calf serum (FCS) (Gibco BRL, Gaithersburg, MD, USA). The cell lines used are listed below along with their American Type Culture Collection (ATCC, Manassas, VA, USA) reference number, or reference. All cells were grown to confluency at 37˚C in a humidified 5 % CO_2 atmosphere.

Name:	Description:	Medium:
A431	human epidermoid carcinoma cells, ATCC: CRL-1555	MEM
A549	human lung adenocarcinoma cells, ATCC: CCL-185	MEM
CHO	Chinese-hamster ovary epithelial cells, ATCC: CCL-61	D-MEM
GC-1 spg	mouse pSV3-neo transformed spermatogonia, (Hofmann *et al.*, 1992)	MEM
HaCaT	immortalized human keratinocytes, (Boukamp *et al.*, 1988)	D-MEM
HT-1080	human fibrosarcoma cells, ATCC: CCL-121	MEM
MDCK	canine kidney epithelial cells, ATCC: CCL-34	MEM

3.1.1 Antibodies

Antigen:	Product nr:	Manufacturer or laboratories:
E-cadherin	HECD-1	Zymed, San Francisco, CA, USA
Epilysin		Lohi *et al.*, 2001; polyclonal rabbit anti-human IgG
HGF	AF-294-A	R&D Systems
LTBP-1	Ab-39	Carl-Henrik Heldin, Ludwig Institute for Cancer Reserch, Uppsala, SWE
LTBP-4		Koli *et al.*, 2005; mouse monoclonal anti-human IgG
Matrilysin		Carole Wilson, University of Washington, Seattle, WA, USA
MT1-MMP	RP1MMP14	Triple Point Biologics, Forest Grove, OR, USA
MT1-MMP		Lehti *et al.*, 2000; polyclonal rabbit anti-human IgG
P-SMAD-2		Peter ten Dijke, The Netherlands Cancer Institute, Amsterdam, NL
Sp1	(1C6) sc-420	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Sp2	(K-20) sc-643	Santa Cruz Biotechnology
Sp3	(D-20) sc-644	Santa Cruz Biotechnology
Sp4	(V-20) sc-645	Santa Cruz Biotechnology
Stromelysin-1	AB19150	Chemicon, Temecula, CA, USA
TGF-β1/LAP		Taipale *et al.*, 1994; affinity-purified rabbit anti-human IgG
TGF-β	AB-100-NA	R&D Systems
V5	R960-25	Invitrogen, San Diego, CA, USA

3.1.2 Enzymes, chemicals and growth factors

All restriction enzymes and ligases were from New England BioLabs (Beverly, MA, USA) unless specified elsewhere. PCR was performed using AmpliTaq Gold™ (Applied Biosystems, Foster City, CA, USA) and RT-PCR using Superscript II RT (Gibco) according to manufacturers' instructions. Recombinant TGF-β and HGF were from R&D Systems (Minneapolis, MN, USA). The synthetic MMP inhibitors BB-3103 and GM6001 were from British Biotech Pharmaceuticals Ltd (Oxford, UK) and Calbiochem (San Diego, CA, USA), respectively. Other chemicals used were Decanoyl-Arg-Val-Lys-Arg-chloromethylketone (CMK) (Bachem, Bubendorf, Switzerland), N-glycosidase F (Roche, Mannheim, Germany) and puromycin (Sigma Chemical Co., St Louis, MO, USA).

3.2 DNA and protein/DNA interaction analysis

3.2.1 Construction of expression vectors

The sequences of expression vectors generated in this work are outlined below, as well as commercial vectors applied. SS denotes signal sequence, PRO denotes prodomain, CAT denotes catalytic domain and PEX denotes hemopexin domain. Mutations in the sequence are denoted by an X.

Name:	Vector:	Insert:	Tag:
		Human epilysin promoter	
pro3031bp	pGL3-Basic	-3031——————————————————————————+1	LUC
pro1486bp	pGL3-Basic	-1486——————————————————————————+1	LUC
pro1167bp	pGL3-Basic	-1167——————————————————————————+1	LUC
pro653bp	pGL3-Basic	-653——————————————————————+1	LUC
pro533bp	pGL3-Basic	-533——————————————————————+1	LUC
pro489bp	pGL3-Basic	-489——————————————————+1	LUC
pro439bp	pGL3-Basic	-439——————————————+1	LUC
pro402bp	pGL3-Basic	-402——————————+1	LUC
pro344bp	pGL3-Basic	-344——————+1	LUC
pro298bp	pGL3-Basic	-298————+1	LUC
pro274bp	pGL3-Basic	-274—+1	LUC
pro1486bpREV	pGL3-Basic	+1——————————————————————————-1486	LUC
pro653bpMUT	pGL3-Basic	-653—X——————————————————————+1	LUC
pro344MUT	pGL3-Basic	-344 X————+1	LUC
		Mouse epilysin promoter	
mpro657bp	pGL3-Basic	-657——————————————————————————+1	LUC
mpro162bp	pGL3-Basic	-162——————————+1	LUC
mpro657bpREV	pGL3-Basic	+1——————————————————————————-657	LUC
		Mouse epilysin cDNA	
		+1 SS I PRO I CAT I PEX +1560	
mEpiS	pcDNA3	+1——————————————————————————+1560	
mEpiM	pcDNA3	+1——————————————————————/ /——————+1560	
mEpiL	pcDNA3	+1——————————————————————/ /————+1560	
pEF-mEpi	pEF-IRESp	+1——————————————————————————+1560	
		Human epilysin cDNA	
		+1 SS I PRO I CAT I PEX +1560	
Epi	pEF-IRESp	+1——————————————————————————+1560	
Epi-E/A	pEF-IRESp	+1————E241A——————————————————————+1560	
Epi*	pEF1/V5-His	+1——————————————————————————+1560	V5
Epi-E/A*	pEF1/V5-His	+1————E241A——————————————————————+1560	V5
Cat*	pEF1/V5-His	+1——————————————————+852	V5
Pex*	pEF1/V5-His	+1——+66 +853——————————+1560	V5

Vector:	Manufacturer:
pBluescriptII KS(+)	Stratagene, La Jolla, CA, USA
pcDNA3	Invitrogen
pCM5-EGFP	Invitrogen
pEF-IRES-P	Hobbs *et al.*, 1998
pEF1/V5-His	Invitrogen
pGEM-4Z	Promega, Madison, WI, USA
pGL3 Basic	Promega
pRL-TK	Promega

3.2.2 Transfections

Cells were washed twice in serum free medium and kept in this media for 24 h prior to transfections. All cells lines, except HaCaT keratinocytes, were transfected using FuGENE 6 (Roche) according to manufacturer's instructions. HaCaT cells on 6-well plates were transfected using 1.5 µg of DNA diluted to 110 µl in Hank's salt solution including 40 µg DEAE-dextran (Promega). Before addition of the DNA-dextran mix, the cells were changed to serum free medium containing 100 µM chloroquine (Promega). After 3 h, the cells were subjected to a 2-min glycerol shock (15 % glycerol in Hanks' salt solution). After transient transfections, cells were incubated in serum free growth medium for 24 or 48 h before analysis.

To generate cell lines with stable, high level expression of different epilysin constructs in the pEF-IRES-P vector, cells were transferred to growth medium containing 1 µg/ml puromycin the day after transfection. During the following 14 days the puromycin concentration was gradually increased to 80 µg/ml (HT-1080, II) or 20 µg/ml (A549, III), selecting a pool of cells with high expression levels of epilysin or the empty vector.

3.2.3 Promoter activity assays

Cells transfected with reporter plasmids containing parts of the epilysin promoter attached to the firefly luciferase gene and the pRL-TK control vector containing the Renilla luciferase gene, were lysed in reporter lysis buffer and analyzed for firefly and Renilla luciferase activities using the Dual-Luciferase Reporter Assay System kit (Promega). Firefly luciferase values were normalized to the renilla value of each sample.

3.2.4 Gel mobility shift assay

For gel mobility shift assays (GMSA) nuclear extracts of HaCaT and GC-1 spg cells were prepared as described (Andrews and Faller, 1991). To determine the binding of nuclear proteins to the GT-box, an oligonucleotide containing this element (5´-GCGGGGTGGGTGGGGCGGGAG-3´) was labeled with [γ-^{32}P]ATP, annealed to its complementary strand, and used as a probe in gel mobility shift assays. Nuclear extracts (6 µg per reaction) were incubated on ice for 20 min in a reaction buffer containing 10 mM HEPES pH 7.9, 60 mM KCl, 1 mM EDTA, 5 mM DTT, 5 mM $MgCl_2$, 10 µM $ZnCl_2$, 10 % glycerol, 4 mM spermidine, 1 µg poly(dI-dC) and 35 fmol labeled probe. For competition experiments, nuclear extracts were preincubated on ice for 30 min with a 100-fold molar excess of unlabeled probe or a mutated version of the probe (5´-GCGGGGTGG**TTT**GGGCGGGAG-3´). Binding to the probe was also competed with a 100-fold molar excess of unlabeled consensus Sp1-oligonucleotide (5´-ATTCGATCGGGGCGGGGCGAGC-3´, Santa Cruz Biotechnology) before addition of the ^{32}P-labeled probe. For supershift assays the nuclear extracts were preincubated with 2 µg of anti human Sp1, Sp2, Sp3 or Sp4 IgG. DNA-protein complexes were then resolved by electrophoresis in a native 4% PAGE in 0.5 X TBE containing 2.5 % glycerol.

3.3 RNA analysis

3.3.1 Extraction of RNA from mouse tissues

Tissue samples were collected from 6-8 week-old, male, NMRI mice. Female reproductive organs were collected from corresponding female mice, and embryos from timed pregnant mice. The samples were frozen in liquid nitrogen. For RNA extraction, the frozen tissues were first disrupted using a mortar and further homogenized in TRIzol® Reagent (Gibco) using a syringe and a 26G needle, and RNA extraction was carried out according to manufacturer's instructions.

3.3.2 RNase protection analysis

Ribonuclease protection assay was used to determine the transcription start site in the human epilysin gene (I), by hybridizing mRNA from HaCaT keratinocytes to an 689 nt [α-^{32}P]UTP labeled anti-sense RNA probe, which covers bases –653 to +1 of the epilysin promoter. To determine the expression of epilysin in different tissues from the mouse (II), total RNA samples were hybridized to a 370 nt [α-^{32}P]UTP labeled anti-sense cRNA probe covering bases 970-1241 of the epilysin cDNA. The probes (100,000 cpm per sample) were hybridized with 10 µg of poly-A RNA or 20 µg of total RNA, respectively. After overnight hybridization unpaired RNA was degraded with a mixture of RNase A and RNase T1 at 37°C for 1 h, followed by isopropanol precipitation (Direct Protect Kit, Ambion Inc., Austin, TX, USA). Protected RNA fragments were separated by polyacrylamide gel electrophoresis in a 5 % TBE-gel containing 6 M urea (Bio Rad, Hercules, CA, USA) and visualized by autoradiography. An antisense cRNA probe complementary to the mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (Ambion) was used as an internal control in comparing the epilysin levels of different tissues. Epilysin- and GAPDH-bands were quantified using a phosphorimager and the epilysin content of each sample was normalized against its GAPDH content (II).

3.3.3 Northern hybridization

A Mouse MTN® blot with 2 µg of mRNA from different mouse tissues was purchased from Clontech (Palo Alto, CA, USA). A 1.5 kb DNA fragment covering the entire coding area of the epilysin mRNA was labeled using [α-^{32}P]dATP and the Strip-EZ DNA™ labeling kit (Ambion). Hybridization was performed in Ultrahyb (Ambion) using 10^7 cpm/ml of labeled probe. After extensive washing of the filter, positive bands were identified by autoradiography (II).

3.4 Protein analysis

3.4.1 Immunofluorescence

Stable cell pools were kept in serum free media 24 h prior to immunofluorescence analysis. Transiently transfected cells were kept for 24 (III) or 48 (II) h after transfection in serum free media before the cells were washed in phosphate buffered saline (PBS) and fixed using 3 % paraformaldehyde (PFA) and permeabilized with 0.1 % Triton X-100. Nonspecific binding sites were saturated with bovine serum albumin (BSA, 5 % solution in PBS), followed by a 1

h incubation with primary antibodies in PBS (II, III). Alternatively, the living cells on glass coverslips were washed with cold PBS and incubated with primary antibodies on ice for 30 min prior to fixation and blocking of nonspecific binding sites (III). For indirect immunofluorescence the cells were incubated with Alexa-conjugated secondary antibodies (Molecular Probes, Eugene, OR, USA), and mounted using VectaShield (Vector Laboratories, Burlingame, CA, USA) containing DAPI to stain the nuclei. Images were obtained using fluorescence microscopy and a digital camera.

3.4.2 SDS-PAGE and immunoblotting

Confluent cell cultures were washed and incubated for 24 (III) or 48 (II) h in serum free MEM. Transiently transfected cells were incubated for 24 h after transfection prior to transferring to serum free medium. The conditioned media were then harvested and cell lysates prepared as described (Lehti *et al.*, 1998). The medium was supplemented with the MMP inhibitor GM6001 (5 µM, final concentration) where indicated. The conditioned media were clarified by centrifugation and concentrated 10-fold using Microcon concentrators (Millipore, Bedford, MA, USA). Aliquots of conditioned medium and cell lysates from the same number of cells were denatured at + 100°C for 3 min in Laemmli sample buffer containing 10 % β-mercaptoethanol (final concentration). The polypeptides of the samples were separated by SDS-PAGE and analyzed by immunoblotting as described (Lohi *et al.*, 2001), using 7.5 % standard or 4-15 % gradient polyacrylamide gels (Bio Rad) and the SuperSignal West Dura Extended chemiluminescence kit (Pierce Chemical, Rockford, CA, USA).

3.4.3 Gelatin zymography

For gelatin zymography the medium polypeptides were dissolved in non-reducing Laemmli sample buffer and separated on 10 % polyacrylamide gels containing gelatin (Invitrogen). The polypeptides were permitted to refold and the gels were stained as described (Lohi *et al.*, 1996).

3.4.4 TGF-β activity assays

Conditioned medium from the same number of cells was analyzed for TGF-β activity either directly (active TGF-β) or after heat treatment (total TGF-β). Heat treatment activates latent forms of TGF-β (Brown *et al.*, 1990). Mink lung epithelial cells stably transfected with a fragment of the PAI-1 promoter fused to the luciferase gene (TMLC) were a kind gift from Dr. Daniel B. Rifkin (New York University School of Medicine, New York, NY, USA). These cells produce luciferase activity in response to TGF-β. TGF-β standards and medium samples were analyzed as described (Abe *et al.*, 1994). The luciferase values were compared to the values of a dilution series of standards containing 7.8 – 500 pg/ml of recombinant TGF-β1. Where indicated, polyclonal antibodies neutralizing the bioactivity of TGF-β and HGF, respectively, were used according to the manufacturer's instructions.

3.5 Transwell migration assays and collagen invasion

Transwell migration assays were performed using type I collagen (0.1 mg/ml; Upstate Biotechnology, Waltham, CA, USA) coated Falcon cell culture inserts with 8 μm pore membranes in 24-well cell culture plates. A549 cells (10^5) in serum free MEM were plated in the upper chambers and MEM supplemented with 0.5 % FCS was added to the lower chambers. The cells were allowed to migrate for 4 h after which they were fixed and stained (40 % methanol, 10 % acetic acid and 0.1 % Coomassie Blue in H_20). Cells were removed from the upper side of the insert, and the migrated cells at the lower side of the membrane were counted. Transiently transfected cells were co-transfected with a construct coding for the enhanced green fluorescent protein (pCM5-EGFP), and transfected cells only were included in the results.

Type I collagen invasion assays were performed essentially as described (Hotary *et al.*, 2000). The collagen solution was neutralized with NaOH and diluted to a final concentration of 2.2 mg/ml in MEM. Collagen gels were then cast into the upper chamber of Falcon cell culture inserts with 8 μm pore membranes in 24-well cell culture plates and allowed to set at +37°C for 1 h. A549 cells (10^6/insert) in MEM containing 10 % FCS were added on top of the gel, and MEM containing 10 % FCS supplemented with 10 ng/ml recombinant HGF to act as a chemoattractant was added to the lower chamber. Where indicated GM6001 (10 μM, final concentration) was added to both chambers. The cells were grown for 8 days changing the media every third day, after which the gels were removed from the inserts, fixed in 3 % PFA, dehydrated and embedded into paraffin. Sections were stained with hematoxylin and eosin (HE-staining), and the invading cells were counted and photographed under a light microscope. Both experiments were repeated three times with similar results.

4 Results

4.1 Characterization of human and mouse epilysin promoters (I)

To analyze the function of the epilysin promoter, fragments of human and mouse genomic DNA containing areas 5'-upstream of the epilysin gene were isolated and sequenced. Comparison of the mouse and human sequences revealed well conserved areas within the promoters, some of which showed homology to known promoter motifs (Fig. 1 in I). No typical TATA-box was, however, detected and transcription start sites in the human *epilysin* gene were therefore determined by RNase protection. Analysis revealed two transcription start sites at 230 and 210 nt from the translation initiation site (Fig. 1 and 2 in I), which is common for genes lacking a TATA-box.

4.1.1 Identification of potential regulatory sites

The human *epilysin* promoter was analyzed for the presence of consensus transcription factor binding sites using internet-based search programs. A GT-box similar to the consensus binding site for transcription factors of the Sp-family was found about 60 bp upstream from the transcription start sites. This motif is completely conserved between the human and mouse genes, implying a possible functional role within the promoter (Fig. 1 in I). Further, about 540 bp upstream from the translation start site in the human promoter a consensus binding site for transcription factors of the SRY/Sox-family was found. This sequence was not conserved in the mouse promoter, where, however, another Sox-like sequence was found close upstream. These two sites were chosen for further analysis as possible regulatory sites within the *epilysin* promoter.

4.1.2 Analysis of transcriptional activity

To elucidate the regulatory effects of the different areas within the epilysin promoter, reporter constructs were generated containing various fragments of the human epilysin promoter coupled to the firefly luciferase gene (constructs illustrated in paragraph 3.2.1). The constructs were transfected into HaCaT keratinocytes and GC-1 spg spermatogonia, which both express endogenous epilysin, and reporter gene transcription was monitored (Fig. 3 in I). The two shortest constructs had negligible effect on reporter gene transcription, whereas the shortest construct including the GT-box, increased the transcriptional activity about 5-fold in both keratinocytes and spermatogonia. To specify whether the GT-box alone was responsible for this induction, a mutation was introduced into it (Fig. 3 in I). The transcriptional activity of the mutated construct was reduced to the level of the shortest constructs, implicating a central role for the GT-box as a binding site for positive regulatory factors.

All longer constructs exhibited high reporter gene activity with some differences between the two cell lines (Fig. 3 in I). The Sox-site in the receptor construct marginally increased the transcriptional activity in keratinocytes, while in spermatogonia the increase was about 40 %. Mutation of the Sox-site gave similar results indicating a cell type specific difference in the regulation of the human epilysin gene, where the putative Sox-site plays a more prominent role in spermatogonia than in keratinocytes. For comparison, constructs containing 657 bp and 162 bp of the mouse promoter sequence, respectively, were generated. In mouse spermatogonia, the 657 bp construct containing the GT-box increased reporter gene transcription markedly, whereas the short construct had no detectable effect on transcription.

4.1.3 The epilysin promoter interacts with Sp1 and Sp3

As the GT-box seemed important for the regulation of the transcriptional activity of the epilysin gene, we next analyzed by GMSA whether any nuclear proteins are bound to this sequence (Fig. 4 in I). The results indicated that nuclear extracts from HaCaT keratinocytes contain proteins that specifically interact with the GT-box in the epilysin promoter, as three distinct protein/DNA complexes could be visualized in gel mobility shift assay. All three complexes were efficiently competed by both an excess of unlabeled GT-probe and Sp1 consensus probe, but not by the mutated GT-probe. The proteins involved in these complexes were identified by supershift assays. Antibodies specific for Sp1 recognized the largest complex, whereas a Sp3-antibody recognized the two smaller complexes. These results indicate that both Sp1 and Sp3 interact with the epilysin GT-box and hence could be responsible for the positive effect on gene transcription mediated through the GT-box.

4.2 Cloning of mouse epilysin cDNA (II)

To clone the mouse homologue of epilysin, a mouse heart cDNA library was screened using a human epilysin cDNA probe. A 1.5-kb fragment was selected for further analysis and sequenced. Comparison of the obtained mouse sequence with the human epilysin cDNA indicated that the fragment contained part of exon 2, exons 3-8, and ~800 bp of 3'-untranslated region (UTR) of the mouse epilysin cDNA. The missing N-terminal sequence was obtained by RT-PCR using RNA from GC-1 spg mouse spermatogonia as a template, a forward primer located in the previously sequenced 5'-UTR of the mouse epilysin gene and a reverse primer in the third exon. When comparing this sequence with the human epilysin cDNA, we found that 30 nt in the beginning of the seventh exon were missing in the mouse cDNA obtained from the cDNA library. To determine whether this sequence represents one of several splice variants expressed in the mouse, we performed RT-PCR with primers on each side of the exon border and RNA from GC-1 spg mouse spermatogonia as a template. In addition to the expected PCR product representing the splice variant found in the library, two other splice variants were identified (Fig. 1 in II). The major form represented full length epilysin, corresponding to the human cDNA, and a minor form represented yet another splice variant with 72 nt missing in the beginning of the seventh exon.

4.2.1 Mouse and human epilysin proteins are highly similar

The full length mouse cDNA contains an open reading frame of 1560 nt from the first ATG codon, coding for a 520 aa protein with a calculated molecular weight of 59 kDa (Fig. 1 in II). The predicted amino acid sequence of mouse epilysin includes several features typical for MMPs. A hydrophobic signal sequence is followed by a propeptide including the characteristic cysteine-switch sequence. In mouse epilysin this sequence (P89RCGVAD95) differs from its human homologue in that it contains an alanine residue instead of a threonine in position 94. The propeptide also contains an YGYL-sequence found in all membrane-bound MMPs, but of the secreted MMPs only in MMP-19. The propeptide ends in a putative furin recognition sequence RKKR. The catalytic center of both human and mouse epilysin, HEIGHTLGLTH, differs from other MMPs in the respect that no other family member contains threonine within this sequence. The catalytic domain is followed by a hinge region and a typical hemopexin domain. Two potential N-glycosylation sites found in the catalytic and hemopexin domains of human epilysin are conserved within the mouse homologue. The detected alternative splicing of the seventh exon affects the hemopexin domain, deleting 10 or 24 amino acids from this domain but preserving the open reading frame (Fig. 1 in II). In the

shortest splice variant the N-glycosylation site within the hemopexin domain is deleted. In mouse epilysin 85 % of the amino acids are identical to the human protein with the highest similarity in the catalytic domain (97 % identical residues).

4.2.2 Epilysin is processed by a furin-like pro-protein convertase

To assess if all three splice variants of mouse epilysin cDNA are translated to proteins, expression constructs coding for the naturally occurring forms of the enzyme were generated and transfected into CHO cells (constructs illustrated in paragraph 3.2.1). Immunofluorescence studies of the transiently transfected CHO cells using epilysin specific antibodies indicated that all three transcripts were translated to proteins that could be visualized in the perinuclear area, presumably in the Golgi apparatus (Fig. 2 in II). Immunoblotting analysis of conditioned media from these cells further showed that all splice variants were secreted to the growth medium. In this transient system epilysin was expressed only as one band of equal size in both growth medium and cell lysates, corresponding to the approximate size of the proenzymes (Fig. 2 in II).

To analyze the secretion and processing of epilysin, we generated a stable HT-1080 fibrosarcoma cell line overexpressing recombinant mouse epilysin. Immunoblotting of the conditioned medium using epilysin-specific antibodies revealed two bands of 48 and 58 kDa, the 48 kDa band being more prominent (Fig. 3 in II). Treatment of the cells with a synthetic furin inhibitor increased the intensity of the 58 kDa form at the expense of the shorter form. Only the 58 kDa form was detected in cell lysates. To investigate whether recombinant mouse epilysin is glycosylated, conditioned medium from cells overexpressing epilysin was treated with N-glycosidase F (Fig. 4 in II). The apparent molecular weight of both the 48 and 58 kDa forms were reduced by enzyme treatment. These results indicating that epilysin is activated intracellularly by furin and that epilysin is N-glycosylated.

4.2.3 Epilysin is widely expressed in different tissues in the mouse

To determine the expression levels of epilysin mRNA in mouse tissues, RNA was extracted from tissues of NMRI-mice and analyzed by RNase protection (Fig. 5 in II). Epilysin mRNA was detected using a cRNA probe covering the exon border between exons six and seven, where alternative splicing occurs, allowing for detection of the full length and intermediate splice variants. Most of the analyzed tissues expressed detectable levels of epilysin mRNA, the highest expression levels being in the lung and the placenta. The other organs expressing moderate levels of epilysin were heart, salivary gland, thymus, uterus, testis, stomach, small intestine, and colon. Expression levels were low in the developing embryo and in the adult liver, kidney and brain. The full length mRNA was the predominant form in all tissues, and the ratio of full length form to the shorter splice variants was constant. Expression levels were low in the developing embryo and in the adult liver, kidney and brain. To determine the size of the mouse epilysin transcript, an epilysin specific cDNA probe was hybridized to a Northern blot containing mRNA from various mouse tissues (Fig. 6 in II). One transcript of about 4.5 kb was detected and, as in the RNase protection analysis, most prominent expression was seen in the lung. These results indicate that epilysin is normally expressed in most tissues in the mouse.

4.3 Epilysin induces epithelial to mesenchymal transition in lung carcinoma cells (III)

To gain more information about the biological functions of epilysin we generated A549 human lung adenocarcinoma cell pools stably overexpressing wild type (wt) human epilysin (Epi) and a catalytically inactive mutant of epilysin (Epi-E/A). During the selection process, when the epilysin content of the cell pools was gradually increased, the morphology of the cells was dramatically altered. The epithelial A549 cells expressing wt epilysin detached from each other, elongated and adopted a fibroblast-like appearance consistent with EMT (Fig. 1 in III). This change of phenotype was repeatedly observed in independent experiments. The cell pools expressing the catalytically inactive mutant of epilysin showed no signs of EMT.

A hallmark of EMT is the loss of the cell surface adhesion protein E-cadherin. No E-cadherin could be detected on the surface of cells expressing wt epilysin that had undergone EMT, but the E-cadherin staining on the cell surface of control and Epi-E/A pools was abundant (Fig. 1 in III). Accordingly, increased levels of the shed 80 kDa ectodomain of E-cadherin were detected in the conditioned medium of epilysin expressing cells. These results indicate that enhanced E-cadherin shedding and loss of E-cadherin mediated cell-cell adhesion coincide with the transition of epilysin expressing A549 cells from epithelial to mesenchymal cell morphology.

4.3.1 TGF-β mediates the epilysin-induced EMT

Because of the well known capacity of TGF-β to initiate and maintain EMT we next determined the levels of active and total TGF-β in the A549 cell pools using the mink lung epithelial cell (Mv1Lu) indicator assay. The levels of both active and total TGF-β were markedly increased in the medium of epilysin expressing cells (Fig. 2 in III). Mature TGF-β remains latent through association with LAP, which can be sequestered to the ECM through association with the LTBPs. Latent TGF-β can be activated by proteolytic processing of the latent complex. To determine whether processing of large latent TGF-β complexes takes place in the epilysin expressing pools, antibodies specific for latent TGF-β1 complexes and for LTBP-1 were used to identify polypeptides from their conditioned medium (Fig. 2 in III). The results revealed only degraded TGF-β1 complexes in the medium from the wt epilysin expressing cell pool, whereas only intact large latent complexes were detected in the medium from the control and Epi-E/A pools. Similarly, LTBP-1 was truncated and digested in the epilysin pool whereas TGF-β containing large LTBP-1 complexes were found in both the control and Epi-E/A pools. Taken together, these results reveal proteolytic degradation of latent TGF-β1/LTBP-1 complexes in cultures of the transformed epilysin expressing pool, evidently resulting in the enhanced secretion and activation of TGF-β.

We next attempted to determine whether the increase in TGF-β activity was the cause or consequence of EMT. For this purpose we selected A549 cell pools stably expressing epilysin in the constant presence of the synthetic MMP inhibitor GM6001. We found that transformation of the epilysin expressing cells could not be reversed by GM6001, but addition of the inhibitor already at the initiation of the selection procedure significantly delayed the epilysin-induced EMT. To further characterize the epilysin-induced EMT these untransformed epilysin expressing cells were changed to medium supplemented with antibodies neutralizing the activity of either TGF-β or HGF or left with GM6001 or without any supplements (Fig. 3A in III). After 72 h, as expected, the TGF-β levels in pools kept with GM6001 were comparable to those of the control and Epi-E/A pools and the cells showed no phenotypic alterations at this point. The cells incubated with neutralizing anti-TGF-β

antibodies similarly retained their normal epithelial morphology and low total TGF-β levels. On the contrary, the pools incubated either without any supplements or with the anti-HGF antibodies revealed an increase in the levels of both active and total TGF-β. These cells also showed alterations characteristic of EMT in their morphology as they started to detach from each other and elongate within 72 h. Next, the cells were stained with antibodies specific for E-cadherin and the phosphorylated form of the TGF-β signaling molecule Smad-2 (P-Smad-2) (Fig. 3B in III). Smad-2 is phosphorylated in response to TGF-β stimulation through TGF-β receptors on the cell surface and is an essential mediator of TGF-β-induced EMT in tumor progression. The results revealed that cells with abundant E-cadherin staining on the surface simultaneously showed weak staining for nuclear P-Smad-2, in contrast to the E-cadherin negative cells that in general showed a more prominent nuclear P-Smad-2 staining. Counting of the E-cadherin positive cells showed more positive cells in the GM6001 and anti-TGF-β treated pools as compared to the cells incubated without any supplements or with the anti-HGF antibodies, whereas the results for P-Smad-2 positive cells were the contrary. These results indicate that neutralizing anti-TGF-β antibodies, like the MMP inhibitor GM6001, can antagonize epilysin-induced EMT in A549 cells revealing TGF-β as a critical mediator of this process.

4.3.2 Induction of gelatinase B and MT1-MMP in epilysin-induced EMT

Given the ability of several MMPs to activate other MMPs, we next determined the levels of stromelysin-1 and matrilysin, whose functions have been linked to the shedding of E-cadherin, in the different cell pools. No stromelysin-1 was immunodetected in the medium from any of the cell pools. A faint band likely to represent matrilysin was detected in all the media, but no major difference could be detected between the different pools (Fig. 4A in III). Thus the epilysin-induced E-cadherin degradation is not likely to involve stromelysin-1 or matrilysin activation or activity. Further, the alternatively spliced form of the small GTPase Rac1 (Rac1b) that underlies stromelysin-1-induced EMT could not be detected by RT-PCR in any of the pools suggesting that the TGF-β dependent EMT mechanism adopted by the epilysin expressing cells is distinct from the one adopted by stromelysin-1. Among MMPs, gelatinases A and B and MT1-MMP have been suggested to mediate TGF-β activation and invasion of transformed cells. Interestingly, both MT1-MMP and gelatinase B were significantly increased in the epilysin expressing cell pool (Fig. 4B and C in III).

4.4 Epilysin is associated with the surface of epithelial cells (III)

To determine how the structure of epilysin is related to its function, we carried out transient transfection experiments of A549 lung adenocarcinoma cells with expression constructs coding for wt epilysin (Epi*), a catalytically inactive mutant of epilysin (Epi-E/A*) and deletion constructs containing either the pro and catalytic domains (Cat*) or the hemopexin domain (Pex*) of epilysin, all with C-terminal V5(*)-tags (constructs illustrated in paragraph 3.2.1). Immunoblotting analyzis of the conditioned medium and cell lysates showed that all the recombinant proteins were detectable in both (Fig. 5B in III). The wt and E/A forms of epilysin were mostly found in the active form but also in the proenzyme form. As expected, the Cat* protein was also detected in both pro and active forms, whereas the Pex* protein migrated as one band.

Although epilysin itself does not contain a transmembrane domain or membrane anchoring sequence, immunofluorescence analysis of the transfected cells showed that epilysin, as well as the Pex* protein and the inactive Epi-E/A* mutant, were localized on the

cell surface (Fig. 5C in III). In contrast, the protein containing only the catalytic domain was not detectable on the cell surface, indicating that the hemopexin domain is sufficient for this interaction. Recombinant epilysin was localized on the cell surface also in the other examined cell lines of epithelial origin, whereas no recombinant epilysin could be detected on the surface of HT-1080 fibrosarcoma cells, suggesting that epilysin is bound to the cell surface through an epithelial cell specific structure.

4.4.1 Cell surface association of epilysin is lost upon EMT

In contrast to the abundant cell surface expression observed in the transiently transfected cells, epilysin was not detected by specific antibodies on the surface of the stable cell pools that had undergone EMT (Fig. 6 in III). The catalytically inactive E/A mutant displayed, however, abundant cell surface expression also in the stable pools. Addition of GM6001 to the transformed wt expressing pool, which did not reverse the transformed phenotype, did not either restore epilysin on the cell surface. These results indicated that either the turnover of epilysin on the surface of the transformed cells was more rapid than that of the Epi-E/A mutant in the cells displaying an epithelial phenotype or that the receptor attaching epilysin to the cell surface was downregulated during EMT.

In transient transfection experiments, the transfection of epilysin with relatively low efficiency (5-10 % transfected cells, Fig. 5 in III) did not result in significant TGF-β induction, and the morphological cell transformation was also less clear than in the wt epilysin expressing stable cell pools. Interestingly, immunofluorescence analysis of A549 cells transiently cotransfected with different epilysin and MT1-MMP constructs showed that all cells expressing MT1-MMP on the cell surface were negative for epilysin (Fig. 5D in III). Coexpression of epilysin and MT1-MMP could, however, be detected on the cell surface in the presence of GM6001 or when the catalytically inactive mutant of MT1-MMP (MT1-E/A) was expressed. These results indicate that either epilysin or its cell surface receptor is destabilized or degraded by MT1-MMP. Indeed, in the progress of EMT the induction of MT1-MMP may cause the observed loss of cell surface bound epilysin.

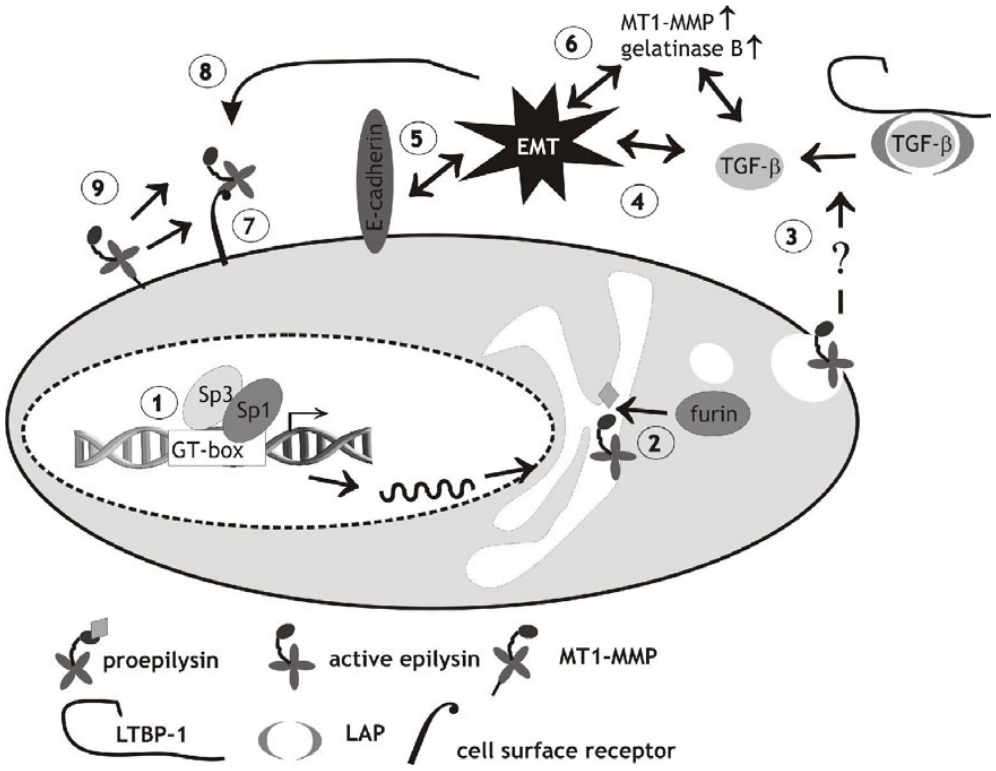
4.5 Epilysin increases the motility of lung carcinoma cells (III)

A characteristic feature of EMT is the increased motility of the transformed epithelial cells. To determine whether epilysin can affect cell motility, transiently transfected A549 cells and stable A549 cell pools were seeded on type I collagen coated cell culture inserts. After 4 h, the cells that had migrated to the lower side of the membrane were fixed and counted. The results showed a marked increase in the migration of both transient and stable epilysin expressing cells over control transfectants (Fig. 7 in III). In contrast, the expression of the catalytically inactive Epi-E/A mutant did not increase the migration of the A549 cells in either case. Interestingly, the MMP inhibitor GM6001 markedly decreased the number of the migrated, transiently transfected, epilysin expressing cells, but did not have a significant effect on the migration of the stable epilysin expressing A549 cell pool. On the contrary, transient transfection of epilysin into HT-1080 cells, which normally display mesenchymal morphology, did not affect their migration. These results suggest that epilysin-induced cell transformation from a quiescent to a motile phenotype rather than cell migration itself requires MMP activity.

4.5.1 Epilysin-induced EMT results in a collagen invasive phenotype

A critical feature of transformed and mesenchymal cells is their ability to invade collagen rich tissues. To define whether the epilysin-induced EMT provides the cells with the means to invade collagen matrices, transiently transfected A549 cells and stable A549 cell pools were seeded on top of type I collagen gels in cell culture inserts. After 8 days in culture, prominent invasion of transformed epilysin expressing cells into the underlying collagen was observed by microscopy of HE-stained paraffin sections (Fig. 8A in III). In contrast, no invading cells were found in the control or Epi-E/A cell pools. The MMP inhibitor GM6001 significantly inhibited the invasion of the transformed epilysin expressing cells. Consistent with the less evident transformation of the phenotype and absence of TGF-β induction in cells transiently transfected with epilysin, only a few of these had invaded into the collagen gel (Fig. 8B in III). The few invaded cells, however, all showed a transformed phenotype as determined by microscopically examining the appearance of the cells. In accordance with the previously identified role of MT1-MMP in collagen invasion, the transiently MT1-MMP transfected A549 cells invaded readily into the collagen gel. Taken together these results show that epilysin-induced EMT provides the cells with collagen invasive properties.

4.6 Central findings of the work



The most important findings of the current study are summarized in Fig. 7.

Figure 7. Summary of the results: Transcription factors Sp1 and Sp3 bind to a conserved GT-box in the epilysin promoter (1); proepilysin is activated by furin (2); overexpression of epilysin leads to the degradation of large latent TGF-β complexes and a subsequent increase in active TGF-β (3) and induction of EMT (4), loss of cell surface E-cadherin (5) and upregulation of MT1-MMP and gelatinase B (6). The two-headed arrows indicate that the internal order and causality of the events involved in the epilysin-induced EMT process is not known. Epilysin is bound to the cell surface by an unknown receptor (7). This cell surface association is lost upon EMT (8) and susceptible to cleavage by MT1-MMP (9).

5 Discussion

5.1 Regulation of *epilysin* gene expression (I)

Individual and stringent regulation of the expression of *Mmp* genes has been pointed out as an important means of targeting the actions of these potent proteases to suitable substrates in the correct location and time frame (Ra and Parks, 2007). To gain knowledge about the regulation of *epilysin* gene expression we have sequenced and characterized the promoters of the human and mouse *epilysin* genes, and compared their structure and function. Computer analysis of the human and mouse *epilysin* promoter sequences revealed several putative binding sites for known transcription factors. Most notably, a GT-box with homology to the consensus binding site for the Sp-family of transcription factors was found about 70 bp upstream from the transcription initiation site. This site is perfectly conserved between the human and mouse promoters, implying a central role for this sequence in the regulation of gene expression. The importance of this GT-box was verified by the fact that mutation or deletion of the GT-box significantly reduced the transcriptional activity of reporter gene constructs in both keratinocytes and spermatogonia. Gel mobility and supershift assays revealed that the transcription factors Sp1 and Sp3 bind this GT-box sequence *in vitro* (I).

5.1.1 Specific regulation of gene expression by Sp1 and Sp3

The Sp-family of transcription factors includes four zinc finger DNA binding proteins, of which Sp1 and Sp3 are ubiquitously expressed in a wide variety of tissues and hence regulate numerous genes including common housekeeping genes. Regulation of gene expression by the Sp-family can, nevertheless, be strictly controlled allowing for cell type specific and cell cycle and developmental stage specific variations in gene expression (Li *et al.*, 2004a). Sp1 and Sp3 are highly homologous and both bind to the same consensus sequence, so called GC- or GT-boxes, in the proximal promoters of target genes (Suske, 1999). Sp1 usually functions as an inducer of transcription whereas Sp3 either induces or represses transcription. The ratio of Sp1 to Sp3 is therefore an important means of regulating transcription, and variations in this ratio can be observed during cell cycle progression and cell differentiation (Li *et al.*, 2004a). During differentiation of primary keratinocytes the initially high Sp3/Sp1 ratio is reversed inducing the transcription of epithelial specific genes such as E-cadherin (Apt *et al.*, 1996), which contains several potential GC/GT-boxes within its promoter (Liu *et al.*, 2005). The cell type and differentiation stage specific regulation of Sp target genes in keratinocytes is of specific interest, as epilysin was originally cloned from a keratinocyte cDNA library and the protein is expressed in primary and transformed keratinocytes (Lohi *et al.*, 2001).

During dermal wound healing, epilysin expression is strongly upregulated in hyperproliferative basal and suprabasal keratinocytes distal from the wound edge (Lohi *et al.*, 2001; Saarialho-Kere *et al.*, 2002). Wound healing of the corneal epithelium is initiated by massive secretion of fibronectin that is later replaced by laminin. During the healing process, these ECM proteins have been implicated in the regulation of the expression of the α5 and α6 integrin subunits through alterations in the levels of Sp1 and Sp3 (Gingras *et al.*, 2003; Gaudreault *et al.*, 2007), suggesting that these factors could be used more widely for coordinating gene expression during wound repair. Differentiation stage specific regulation of gene transcription by Sp1 is also crucial in male germ cell development (Thomas *et al.*, 2007), and in embryogenesis (Marin *et al.*, 1997). In view of data on gene regulation by Sp1 and Sp3 it appears likely that the regulation of the *epilysin* promoter through its Sp1/Sp3 binding GT-

box may be of central importance for both the basal and cell type specific expression of epilysin.

5.1.2 Sp1 and Sp3 are mediators of TGF-β-induced gene regulation

Sp1 and Sp3 regulate gene expression by functionally cooperating with other proteins, such as other transcription factors and chromatin remodeling factors (Li *et al.*, 2004a). Interestingly, Sp1 interacts directly with transcriptional regulators of the Smad-family, an association that is induced by TGF-β signaling and results in enhanced DNA binding. Sp1 has thus been recognized as an important factor in the induction of many TGF-β regulated genes (Moustakas *et al.*, 2002). The ratio of Sp1 to Sp3 also critically regulates the expression of TGF-β receptor II, as Sp1 enhances while Sp3 represses its expression. Overexpression of Sp3 has therefore been indicated as one mechanism adopted by different cancer cells to circumvent the growth inhibitory effects of TGF-β (Safe and Abdelrahim, 2005). Interestingly, a recent report proposed that Sp1, in cooperation with activated Smads, is a crucial mediator of TGF-β-induced EMT in pancreatic cancer cells, as abrogation of Sp1 in this cell system prevented migration but not proliferation of the transformed cancer cells (Jungert *et al.*, 2007). As we have found that epilysin induces EMT in lung carcinoma cells through mechanisms depending on TGF-β and involving the activation of Smad-2 (III), these observations raise the question whether TGF-β is involved in the regulation of the epilysin gene through its functional Sp1/Sp3-binding site (I) and whether epilysin could function both as an inducer and effector of TGF-β signaling. This assumption is supported by the report of a slight induction of epilysin expression detected by real time PCR in primary keratinocytes treated with TGF-β1 (Saarialho-Kere *et al.*, 2002).

Another link between epilysin and TGF-β in EMT involving Sp1 was revealed by studies concerning the transcription factor Snail. Snail is a critical regulator of EMT (Barrallo-Gimeno and Nieto, 2005) and the most important downstream target of TGF-β signaling in EMT (Peinado *et al.*, 2003). Snail induces EMT through numerous target molecules (De Craene *et al.*, 2005), most importantly through repressing the expression of the cell-cell adhesion molecule E-cadherin (Batlle *et al.*, 2000; Cano *et al.*, 2000). Among the MMPs, *in vitro* induction of gelatinase A, matrilysin and MT1-MMP by Snail have been reported (Yokoyama *et al.*, 2003; Miyoshi *et al.*, 2004). When overexpressed in MDCK cells, Snail induces expression of gelatinase B as part of the EMT process (Jorda *et al.*, 2005). Snail does not seem to interact directly with the gelatinase B promoter, but the binding of Sp1 to a specific GC-box within the promoter is required for induction.

5.1.3 Histone deacetylation inhibitors affect epilysin expression

Histones are nuclear proteins involved in the packaging of DNA in the eukaryotic nucleus. The histones can be modified by regulating their state of acetylation by histone acetyltransferases (HATs) and histone deacetylases (HDACs) that reversibly add and remove acetyl groups from their specific lysine residues. Acetylation neutralizes the positive charge of the histones, thereby loosening the histone-DNA interaction and allowing access of transcription factors. In addition, the activity of many other proteins such as structural proteins, intracellular signaling molecules and transcription factors is modulated by acetylation (Clark *et al.*, 2007). It has recently been proposed that epilysin is one of a small subset of *Mmp* genes that is induced by treatment with HDAC inhibitors, in contrast to most other *Mmp* genes that are repressed by these inhibitors (Young *et al.*, 2005). Using our promoter constructs, Swingler *et al.* (submitted, 2007) reported that this induction depends on the GT-box we identified (I). They proposed that overexpression of Sp1, but not Sp3, induces

promoter constructs containing the GT-box *in vitro*, and that this induction is further promoted by HDAC inhibitors. Sp1, rather than histones, may in this experimental setup be the target affected by the HDAC inhibitors, as the activity of Sp1 is regulated by acetylation of the transcription factor itself (Li *et al.*, 2004a). By chromatin immunoprecipitation it was found that Sp1 associates with the GT-box *in vivo*, and by oligonucleotide pulldown experiments association of the serine threonine receptor associated protein (STRAP) with the GT-box in the epilysin promoter was further detected (Swingler et al., submitted 2007). STRAP associates with both type I and II TGF-β receptors and inhibits TGF-β-induced transcriptional responses in synergy with Smad-7 (Datta *et al.*, 1998; Seong *et al.*, 2007). These findings provide yet another link between TGF-β and the regulation of epilysin expression.

Other *Mmp* genes including binding sites for Sp1/Sp3 in their promoters are gelatinases A (Huhtala *et al.*, 1990) and B (Huhtala *et al.*, 1991) and MT1-MMP (Lohi *et al.*, 2000). The *Timp-2* gene also contains binding sites for Sp1/Sp3 (Hammani *et al.*, 1996) as does the gene coding for the cell surface-bound MMP inhibitor RECK (Sasahara *et al.*, 1999). Of these, gelatinase A is repressed whereas TIMP-2 is induced by HDAC inhibitors. The expression of gelatinase B is either repressed or induced, depending on the cellular context (Clark *et al.*, 2007). The expression of RECK is, like epilysin, repressed by HDACs, a repression mediated through the Sp1 binding site (Chang *et al.*, 2004). These observations highlight the possibility to modify the regulation exerted by Sp1/Sp3 on *Mmp* gene expression by acetylation, and further emphasize the importance of the Sp1/Sp3 binding site in the epilysin promoter. However, all these studies have been conducted at least partly *in vitro*, and many other factors may influence the transcriptional activity *in vivo*.

5.2 Epilysin is highly conserved between species (II)

Information on the specific tissues and circumstances under which a proteinase is expressed is essential to determine its function. After all, simply characterizing the enzyme's ability to cleave possible substrates *in vitro* does not necessarily reflect a relevant biological function. To explore the *in vivo* expression and to gain insight into the biological functions of epilysin, we cloned the mouse homologue of the epilysin cDNA and characterized its expression in a variety of tissues. The predicted mouse protein is highly similar to the human enzyme, including the "cysteine switch" sequence in the prodomain, the conserved furin recognition sequence, and the unique catalytic site sequence (II). Between the human and mouse proteins, 85 % of the overall amino acid residues and 97 % in the catalytic domain, are identical indicating a well-conserved function for this enzyme. This assumption is further supported by the observation that epilysin is well conserved also in *Xenopus laevis*, as 56 % of the overall amino acids in XMMP-28 and 65 % in the catalytic domain are identical with human epilysin (Werner *et al.*, 2007). We observed that the potential furin recognition motif at the end of the prodomain is functional, proposing intracellular cleavage of epilysin by the proprotein convertase furin as an important point of regulating enzyme activity. The furin site is also conserved and functional in XMMP-28 (Werner *et al.*, 2007). All the MT-MMPs are activated by furin, whereas stromelysin-3 is the only other furin-activated soluble MMP.

We identified an YGYL sequence in the prodomain of epilysin that is conserved in the human as well as the *Xenopus* protein. Interestingly, a corresponding sequence in the prodomain of MT1-MMP has been proposed to confer the prodomain the capacity to serve as a chaperone regulating the folding and trafficking of the enzyme (Cao *et al.*, 2000; Pavlaki *et al.*, 2002). This sequence is common to all MT-MMPs, but among the other secreted MMPs only MMP-19 contains this sequence (Cossins *et al.*, 1996; Pendas *et al.*, 1997). There are,

however, no reported functions for the YGYL sequence in other MMPs apart from MT1-MMP or in any *in vivo* model, rendering the relevance of this sequence in the epilysin prodomain uncertain.

We further detected alternative splicing of the epilysin mRNA, producing two shorter in-frame splice variants with 10 and 24 amino acids missing from the hemopexin domain, respectively. Alternative splicing affects the second blade of the four-bladed-propeller-like hemopexin domain, and may thus affect its substrate interactions. The splice variants were detected also in tissues from NMRI mice, but the ratio of shorter splice variants to full length mRNA was constant and low, excluding the possibility for tissue specific functions of these splice variants. No corresponding variants of the human mRNA could be detected further questioning the relevance of alternative splicing in the regulation of epilysin function *in vivo*. Another form of alternative splicing of the human epilysin mRNA has, however, been detected generating a shorter mRNA where part of the catalytic domain is missing. In general, alternative splicing of MMPs is an unusual feature as there are only a few reports on this phenomenon (MT3-MMP, Matsumoto *et al.*, 1997; collagenase-2, Hu *et al.*, 1999; stromelysin-3, Luo *et al.*, 2002).

5.2.1 Epilysin is expressed in normal tissues

Tissue expression of epilysin in adult, healthy NMRI mice was determined by RNase protection and Northern hybridization. Epilysin mRNA was detected in various tissues with highest expression levels in the lung. Epilysin mRNA was also detected in the placenta, heart, uterus, testis and gastrointestinal tract. Expression levels were low in the developing embryo and in the adult liver, kidney and brain. In addition to our observations, there is an other report on the expression of epilysin mRNA in mouse embryos (Werner *et al.*, 2007), where the authors report a peak of epilysin expression at E14 in the developing neural system, in both the CNS and peripheral nerves. By *in situ* hybridization epilysin mRNA was detected in the rhesus monkey uterus during early pregnancy (Li *et al.*, 2003a). The tissue distribution of mouse epilysin mRNA resembles that of human epilysin with one notable exception: in adult human tissues the most prominent expression was found in the testis (Lohi *et al.*, 2001), whereas the overall expression levels in the adult mouse testis were only moderate. The wide expression of epilysin is in agreement with the induction of the epilysin gene by the common transcription factor Sp1. This expression pattern, however, distinguishes epilysin from most other MMP-family members, as most MMPs are expressed only under exceptional conditions such as those related to injury, inflammation or disease (Ra and Parks, 2007). The expression pattern implicates a role for epilysin in normal tissue homeostasis and turnover and thus sets a basis for the future efforts to characterize its biological functions and substrates.

5.2.2 Matrilysin, MMP-19 and epilysin are epithelial MMPs

Epilysin is expressed solely by epithelial cells in human skin (Lohi *et al.*, 2001; Saarialho-Kere *et al.*, 2002), and in the mouse uterus epilysin mRNA is expressed exclusively in the endometrium (Illman, S.A., unpublished observation). It is therefore reasonable to speculate that epilysin is expressed in the epithelial compartment also in other tissues. Two other MMPs that have been detected in intact healthy epithelia are matrilysin and MMP-19. Further, stromelysin-2 is expressed exclusively in epithelial cells, but only in response to injury or in carcinomas and not under normal conditions (Saarialho-Kere *et al.*, 1994; Madlener *et al.*, 1996). Matrilysin is expressed in several mucosal epithelia, such as the small intestine, lung and exocrine glands, but not in the epidermis (Saarialho-Kere *et al.*, 1995; Wilson *et al.*, 1995). MMP-19, on the contrary, is expressed in basal keratinocytes in the epidermis (Impola *et al.*, 2003; Sadowski *et al.*, 2003b) and in several other tissues (Pendas *et al.*, 1997).

Matrilysin functions in important processes involved in protecting epithelial tissues (Parks *et al.*, 2004), such as in the defense against bacteria in the intestine (Wilson *et al.*, 1999) and in re-epithelialization in the process of healing lung injury (McGuire *et al.*, 2003). These are indeed biologically relevant functions for matrilysin, since both processes are defective in matrilysin deficient mice. Matrilysin is, however, also involved in the progress of cancer *in vivo* and is frequently detected in cancers of almost all human organs (Wielockx *et al.*, 2004).

MMP-19, on the other hand, has been detected in proliferating but not in invasive SCC cells (Impola *et al.*, 2003; 2005), findings that are further supported by a report on the expression of MMP-19 at the invasive front in early but not later stages of SCC carcinomas (Sadowski *et al.*, 2005). Similarly, MMP-19 is expressed in the normal epithelium of both the mammary gland (Djonov *et al.*, 2001) and colon (Bister *et al.*, 2004), but is downregulated in both tissues upon malignant transformation. In dermal wounds, MMP-19 is detected in keratinocytes outside the migrating area (Impola *et al.*, 2003). MMP-19 increases cell proliferation, migration and adhesion to collagen I *in vitro* through the cleavage of IGFBP-3 and subsequent release of IGF (Sadowski *et al.*, 2003a). Further, MMP-19 is downregulated by transcription factors Tst-1 and Skn-1 that promote keratinocyte differentiation and decrease proliferation (Beck *et al.*, 2007). The generation of MMP-19 deficient mice has, however, revealed an unexpected function for this protease in the regulation of adipogenesis, as MMP-19 deficient mice develop diet-induced obesity (Pendas *et al.*, 2004). No effects on IGF release could, however, be detected in this model. The effect of MMP-19 on cancer progression is controversial. MMP-19 deficient mice develop fewer fibrosarcomas in their skin after treatment with carcinogens (Pendas *et al.*, 2004), whereas tumor angiogenesis and invasion are promoted in the same mice when compared to wt (Jost *et al.*, 2006). These results may reflect the different functions of MMP-19, and MMPs in general, in different forms of cancer and in different stages of tumor progression (Martin and Matrisian, 2007).

The role of epilysin in cancer also appears contradictory, as there are reports on both increased and decreased epilysin expression in different tumors (section 1.2.3.2). A functional role for epilysin has, however, been described in OSCC, where the expression of epilysin mRNA was markedly upregulated (Lin *et al.*, 2006). Secretion of epilysin from OSCC and esophageal carcinoma cells was reduced by capturing epilysin mRNA with antisense oligodeoxynucleotides, which impaired the ability of these cells to form colonies in soft agar without affecting cell growth. These results indicate a role for epilysin in the anchorage-independent growth of both OSCC and esophageal carcinomas. The effect of downregulating epilysin on the invasive capacity of these cells was not determined (Lin *et al.*, 2006). However, in another study, epilysin immunoreactivity was detected in proliferating basal and suprabasal keratinocytes in grade I SCC from the skin, but not in aggressive cutaneous sclerosing basal carcinomas or SCCs of grades II and III (Saarialho-Kere *et al.*, 2002). Epilysin, like MMP-19, thus seems to be involved in cancer cell proliferation in early stages of SCC and downregulated in invasive higher grade carcinomas. Similar observations have been made in colon carcinomas (Bister *et al.*, 2004) and melanomas (Kuivanen *et al.*, 2005), whereas upregulation of epilysin expression in invasive carcinomas of the breast (Overall *et al.*, 2004) and bladder (Wallard *et al.*, 2006) have been observed. Epilysin may, therefore, like MMP-19 play different roles in cancers of different tissues and at various stages of cancer progression.

Another similarity among the epithelial MMPs is that they are associated with the cell surface through interactions with different cell surface receptors. Matrilysin associates with heparan sulfates (Yu and Woessner, 2000; Yu *et al.*, 2002), cholesterol (Yamamoto *et al.*, 2006) and the tetraspanin CD151 (Shiomi *et al.*, 2005), whereas the cell surface receptor for MMP-19 is unknown (Sadowski *et al.*, 2005). We have observed that epilysin associates with

a so far uncharacterized MT1-MMP sensitive cell surface receptor (III), illustrating the importance of compartmentalization for proper MMP function.

5.2.3 Targeted disruption of the epilysin gene in the mouse

Despite the similar expression patterns of the epithelial MMPs, matrilysin, MMP-19 and epilysin all seem to display distinct functions. To further characterize the specific role of epilysin in this triad, we have recently generated a mouse model where the epilysin gene has been disrupted. Initial observations show that the mice breed normally and are born with no obvious phenotype. When its lungs were challenged by different inflammatory agents the epilysin mRNA levels were markedly downregulated in the wt mice (Parks, W.C., personal communication, 2007). The KO mice, on the contrary, show a rapid influx of macrophages into the lungs after infection and altered levels of cytokines and chemokines. The results implicate that epilysin may serve a function in the immune response, an assumption that is supported by the reported induction of epilysin expression in primary keratinocytes by the pro-inflammatory cytokine TNF-α (Saarialho-Kere *et al.*, 2002). Interestingly, a recently cloned MMP from the lepidopteran model *Galleria mellonella* has been implicated in the activation of innate immunity in this organism. This MMP, named Gm1-MMP, shares highest similarity with human MMP-19 and epilysin (Altincicek and Vilcinskas, 2006; 2007).

Among the epithelial MMPs also matrilysin is importantly involved in many aspects of the immune system (Parks *et al.*, 2004) and mice deficient in stromelysin-2 develop much more severe pneumonia upon bacterial infection (Kassim *et al.*, 2007). The healing of skin wounds of epilysin deficient mice appears normal and no abnormalities in either wound closure rate or wound histology have been revealed (Illman, S.A., unpublished observations). A possible role for epilysin in wound healing is cannot be excluded as other MMPs may compensate for the lack of epilysin.

5.3 Epilysin induces TGF-β mediated EMT in lung carcinoma cells (III)

Considering the detection of epilysin in the epithelia of several intact, healthy tissues (Lohi *et al.*, 2001; II) our unexpected observation that the expression of epilysin causes stable EMT in A549 lung adenocarcinoma cells (III) points out a novel role for epilysin in the regulation of epithelial cell phenotype. Consistent with this epithelial cell specific function, a similar phenomenon was observed also in MDCK kidney epithelial cells, whereas epilysin expression caused no observed effects on the morphology and migration of HT-1080 fibrosarcoma cells. Interestingly, an inactivating point mutation of epilysin (E241A), the MMP inhibitor GM6001, and neutralizing antibodies against TGF-β all prevented the onset of EMT, indicating that this is a TGF-β mediated process triggered by the catalytic activity of epilysin. The epilysin-induced EMT was irreversible as the altered phenotype was maintained despite the subsequent addition of the MMP inhibitor.

Members of the TGF-β family can induce and maintain EMT in a variety of biological and pathological systems like wound healing, cancer and fibrosis (Zavadil and Böttinger, 2005). Interestingly, we observed proteolytic degradation of latent TGF-β complexes accompanied by increased levels of both active and latent TGF-β in the morphologically altered epilysin expressing cell pool. These changes were accompanied by increased phosphorylation of the TGF-β signaling molecule Smad-2, an event suggested to be crucial in TGF-β-induced EMT in cancer progression (discussed in Lee *et al.*, 2006). Neutralizing anti-TGF-β antibodies, as well as the MMP inhibitor GM6001, prevented the epilysin-induced

release of active TGF-β and soluble TGF-β complexes and hence prevented EMT in the overexpressing cell pools.

In accordance with increased TGF-β signaling, the mRNA levels of the transcription factor Snail were upregulated about 1.5-fold in the epilysin expressing cell pool. Snail is a downstream target of TGF-β signaling (Peinado *et al.*, 2003) and a critical regulator of EMT (Barrallo-Gimeno and Nieto, 2005). Snail induces EMT through numerous target molecules (De Craene *et al.*, 2005). Among these we found upregulation of gelatinase B (Jorda *et al.*, 2005) and MT1-MMP (Miyoshi *et al.*, 2004) in the morphologically altered cells. By contrast, the expression of gelatinase A was downregulated in these cells further highlighting the extensive change in the MMP expression profile upon this transcriptional EMT program. Interestingly, both gelatinase B and MT1-MMP have the ability to activate TGF-β, at least *in vitro*, by direct cleavage of the LAP complex (Yu and Stamenkovic, 2000; Mu *et al.*, 2002). Further investigations aiming at the identification of direct epilysin substrates will illuminate the more exact cascade of events leading to TGF-β activation and EMT. Current results, nevertheless, indicate that transient activity of epilysin is sufficient to induce a coordinated TGF-β dependent program leading to the loss of the epithelial phenotype and to the gain of characteristics of invasive cancer cells.

With some analogy to these results stromelysin-1 can also initiate EMT (Lochter *et al.*, 1997; Radisky *et al.*, 2005), and both stromelysin-1 and matrilysin can cleave E-cadherin (Noë *et al.*, 2001), which is a critical event in the onset of EMT (Thiery, 2002). In contrast to our results, however, stromelysin-1-induced EMT is mediated by Rac1b and ROS, but does not involve TGF-β. Therefore, even though we find here that the epilysin-induced EMT also involves E-cadherin shedding, the mechanism by which epilysin induces EMT differs from that of stromelysin-1. Further, we observed that the epilysin-induced EMT does not involve the activation of either stromelysin-1 or matrilysin. MT1-MMP and gelatinase B, on the other hand, have both been shown to mediate the shedding of E-cadherin in ischemic kidney NRK cells (Covington *et al.* 2006) and epithelial ovarian carcinoma cells (Symowicz *et al.*, 2007), respectively. As we detected induction of both of these MMPs upon epilysin-induced EMT (III), MT1-MMP and gelatinase B may be responsible for the observed E-cadherin shedding. Interestingly, recombinant XMMP-28 (the epilysin homologue in *Xenopus laevis*) can digest another cell adhesion molecule, the synaptic membrane glycoprotein NCAM, *in vitro* (Werner *et al.*, 2007).

5.3.1 Epilysin induces epithelial cell motility

MMPs exhibit a considerable overlap in their *in vitro* substrates. *In vivo* substrate specificity is determined by the regulation of their affinity towards different substrates and by strict temporal and spatial regulation of the active proteases, so called compartmentalization (Ra and Parks, 2007). Even though most MMPs are secreted as soluble molecules, many of them can interact with specific receptor molecules on the cell surface to direct their proteolytic activity to specific sites in the pericellular environment (Brooks *et al.*, 1996; Dumin *et al.*, 2001; Yu and Stamenkovic, 1999; Yu and Woessner, 2000; Yu *et al.*, 2002). Current data indicate that epilysin, though lacking a transmembrane domain, associates with the surface of epithelial cells through mechanisms involving the hemopexin domain. Accordingly, high molecular weight complexes containing epilysin can be detected on the surface of A549 cells that have not undergone EMT yet (Illman, S.A., unpublished observation). These results identify spatial targeting of epilysin as a novel means of regulating its activity and bringing it into close proximity of potential cell surface targets in epithelial cells.

Directed pericellular proteolysis is crucial in cell migration and invasion through the ECM (Murphy and Gavrilovic, 1999). The morphologically altered A549 cells stably

overexpressing epilysin gained enhanced ability to migrate through type I collagen coated membranes. However, the enhanced migration was not inhibited by GM6001 indicating that epilysin is required only transiently for the epithelial cells to acquire the migratory phenotype, whereas the transwell migration itself was independent of MMP activity. Invasion of both fibroblasts and tumor cells into cross-linked collagen gels *in vitro* as well as into the chicken chorioallantoic membrane *in vivo*, on the other hand, are both processes dependent on the catalytic activity of MT1-MMP (Hotary *et al.*, 2000; Sabeh *et al.*, 2004). However, invasion of carcinoma cells into *in vivo* BMs obtained from mouse peritoneum can be driven by the catalytic activity of either MT1-, MT2-, or MT3-MMP (Hotary *et al.*, 2006). We found that A549 cells transiently expressing MT1-MMP invaded readily into collagen gels, as did the stable epilysin expressing cells that had undergone EMT. The collagen invasion of all cells was inhibited by GM6001 emphasizing the role of MMPs in the process. It has not been determined yet whether epilysin itself possesses any collagenolytic activity. However, as we observed upregulation of MT1-MMP in the transformed cells, the invasive properties can, in agreement with previous observations (Hotary *et al.*, 2000; 2006; Sabeh *et al.*, 2004), most likely be ascribed to this protease. Further, collagenases 1 and 3, which are induced by TGF-β in SCC cells (Leivonen *et al.*, 2006), might also contribute to the invasion of the transformed A549 cells.

Given the well established and important role of cell surface targeting of proteinases for cell invasion (Murphy and Gavrilovic, 1999; Hotary *et al.*, 2000; Sabeh *et al.*, 2004), the loss of pericellular localization of epilysin in the morphologically altered and invasive epilysin expressing A549 cells also supports a less significant role of epilysin activity in collagen invasion. Epilysin would thus regulate cell invasion in an indirect fashion through the induction of other MMPs. Current observations reveal a unique role for epilysin in the regulation of epithelial cell behavior through the activation of TGF-β, induction of EMT and acquirement of a type I collagen invasive phenotype.

5.3.2 Implications for epilysin function *in vivo*

Current results illustrate a dramatic effect for epilysin on the phenotype of epithelial cells. These results were obtained by overexpressing epilysin *in vitro* in lung carcinoma cells. Considering the wide expression of epilysin in normal, healthy tissues an effect of this magnitude on epithelial cell morphology is unlikely *in vivo*. In a physiological setting the circumstances would be quite different as epilysin expression would be regulated by its endogenous promoter. This would allow specific interactions with transcriptional regulators that probably generate much lower expression levels of this enzyme. The cells would also be surrounded by a physiological ECM which presents growth factors, protease inhibitors and other proteases that affect the phenotype of the cells and possibly directly modulate epilysin itself. Further, the A549 cells used in the experimental setup are carcinoma cells which do not respond to extracellular signals in the same way as normal cells do.

In several experimental setups where epilysin has been overexpressed under different promoters and selection markers, only cells with low epilysin levels have survived and by time most cells have lost epilysin expression (Illman S.A., unpublished observations). In the current cell system we took advantage of the pEF-IRES-P vector (Materials and Methods), which expresses the inserted cDNA and a puromycin resistance gene as a single mRNA molecule with an internal ribosomal entry site in between. Using this vector, all selected puromycin resistant cells are forced to maintain the expression of epilysin. In this way we succeeded in maintaining the epilysin expression at high levels, inducing EMT in the A549 cells (III). In light of these results it is possible that high levels of epilysin are toxic to cells, and that only a small subset of cells can survive and adapt to the high epilysin contents

driving them into EMT. This may also explain why the regulation of epilysin expression varies between cancers of different tissues and different stage of carcinogenesis (section 1.2.3.2).

Nevertheless, these results indicate a possible interaction between epilysin and TGF-β also under physiological circumstances. *In vivo* epilysin activity may not induce EMT but, instead, trigger less permanent changes in TGF-β signaling and cell motility, for example when it is upregulated in basal skin keratinocytes during wound healing (Lohi *et al.*, 2001; Saarialho-Kere *et al.*, 2002). Interactions between epilysin and TGF-β may also be of importance in regulating the functions of the immune system, as indicated by the increased inflammatory response observed in epilysin deficient mice (section 5.2.3). Epilysin expression has recently been reported in the developing nervous system in mouse and *Xenopus* embryos and also in regenerating nerves in *Xenopus*, where the neural adhesion protein NCAM was identified as an *in vitro* substrate for epilysin (Werner *et al.*, 2007). Considering the timing of epilysin expression during development and nerve regeneration, the authors speculate that epilysin could be involved in altering the neural microenvironment leading to the development of myelin or in inducing a more motile phenotype in the glial cells. These results support our observation that epilysin can induce the motility of epithelial cells, and imply that epilysin could modulate the adhesive properties not only of epithelial cells but also of neural cells through directed proteolysis of cell surface molecules.

6 Perspective

The generation of genetically targeted mice has provided new insights into the biology of MMPs. For many MMPs, the relevant *in vivo* substrates and functions have turned out to be quite different from what had been expected in view of previous *in vitro* data. Nevertheless, studies on these mice have provided proof for the crucial involvement of the MMPs in development, in the regulation of the immune system and innate immunity, in wound healing and in pathological processes such as the induction and progression of carcinogenesis and in arthritis (Egeblad and Werb, 2002; Parks *et al.*, 2004; Martin and Matrisian, 2007; Page-McCaw *et al.*, 2007).

Future research concerning the individual role for epilysin in the MMP family should be focused on *in vivo* experimental systems. Current data elucidate important aspects on the regulation of epilysin activity through its promoter, activation of the proenzyme and targeted localization to the cell membrane of epithelial cells. The important finding that epilysin can induce EMT in epithelial cells *in vitro* through TGF-β dependent mechanisms, will guide further research to elucidate the involvement of epilysin in promoting cell motility in TGF-β regulated processes, such as inflammation and wound healing. The genetically targeted mouse strain lacking the epilysin gene will be a valuable asset in this pursuit, even though deletion of genes coding for individual soluble MMPs has not generated severe phenotypes in mouse models reported so far. In response to various challenges these mice have, however, frequently displayed individual phenotypes revealing biologically relevant functions and substrates for the MMPs. It is therefore reasonable to speculate that some of the biologically relevant functions of epilysin are in the response cascades activated by specific challenges.

One disadvantage with the epilysin null mice is that they have been lacking epilysin ever since conception, and other MMPs may be upregulated to compensate for the loss of its enzymatic activity. Challenges inflicted on these mice will therefore reveal only the reactions of an organism adapted to the lack of epilysin. The generation of mice with a tissue specific deletion of the epilysin gene could therefore provide additional clues to the function of epilysin. Conditional inactivation of the epilysin gene in the skin of adult mice could, for example, reveal a role for epilysin in wound healing without the inflicting compensation by other MMPs. Specific downregulation of epilysin in both time and space could also be achieved through the use of siRNA designed to interfere with epilysin mRNA. Further knowledge on the biology of epilysin will elucidate if targeted inactivation of this enzyme could be beneficial in the treatment or prevention of specific diseases. On the contrary, enhancement of epilysin function could possibly promote resistance to and recovery from injury and disease.

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